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Resistance Mechanisms of Non-Hodgkin Lymphomas against Rituximab Treatment

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ACADEMIC DISSERTATION

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2. **Harjunpää A**, Wiklund T, Collan J, Janes R, Rosenberg J, Lee D, Grillo-López A, Meri S. *Complement activation in circulation and central nervous system after rituximab (anti-CD20)* treatment of B-cell lymphoma. Leukemia and Lymphoma. 42:731-738, 2001.

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*)Equal contribution

ABBREVIATIONS

ADAM10	A disintegrin and metalloproteinase 10
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myelogenous leukemia
AP	Alternative pathway
APC	Antigen-presenting cell
APC-conjugated	Allophycocyanin-conjugated
ARDS	Adult respiratory distress syndrome
C	Complement
C1qR	C1q-receptor
C4bp	C4-binding protein
C9DS	C9-deficient serum
CD20cy	CD20-antibody reacting with cytoplasmic epitopes
CD35	Complement receptor 1
CD46	Membrane cofactor protein
CD55	Decay accelerating factor
CD59	Protectin
CDC	Complement-dependent cytotoxicity
CDCC	Complement-dependent cellular cytotoxicity
CHOP	Cyclophosphamide, doxorubicin, vincristine, and prednisone
CLL	Chronic lymphatic leukemia
CNS	Central nervous system
CP	Classical pathway
CR	Complete response
CR1	Complement receptor 1 (CD35)
CSF	Cerebrospinal fluid
CVP	Cyclophosphamide, vincristine and prednisone
DAF	Decay accelerating factor (CD55)
DC	Dendritic cell
DLBCL	Diffuse large B-cell lymphoma
EGFR	Epidermal growth factor receptor
EphA1	Ephrin receptor A1
ERK1/2	Extracellular signal-regulated kinases 1/2
FcR	Fc-receptor
FFS	Failure free survival
fH	Factor H
fI	Factor I
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
FLIPI	Follicular Lymphoma International Prognostic Index
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycosyl-phosphatidyl-inositol (anchor)
HDT/ASCT	High-dose chemotherapy with autologous stem cell transplant
HIS	Heat-inactivated serum
HR	Hazard ratio

HER2	Human epidermal growth factor receptor 2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPI	International Prognostic Index
IR	Immune response
LAK	Lymphokine-activated kiIler
LDH	Lactate dehydrogenase
LP	Lectin pathway
mAb	Monoclonal antibody
MAC	Membrane attack complex
MALT	Mucoid associated lymphoid tissue
Marco	Macrophage receptor collagenous structure
MAPK	Microtubule-associated protein (MAP) kinase
MASP	Mannan-binding lectin associated serine protease
MBL	Mannan-binding lectin
MCL	Mantle cell lymphoma
Mcl1	Myeloid cell leukemia sequence 1
MCP	Membrane cofactor protein (CD46)
MHC	Major histocompatibility complex
MS4A1	Membrane-spanning 4-domain, group A, member 1 (CD20)
N-FMLP	N-formylmethionyl-leucylphenylalanine
NHL	Non-Hodgkin lymphoma
NHS	Normal human serum
NK cell	Natural killer cell
OR	Overall response
OS	Overall survival
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll
PFS	Progression free survival
PMA	Phorbol myristate acetate
PKC	Protein kinase C
RKIP	Raf-kinase inhibitory protein
qPCR	Quantitative polymerase chain reaction
R	Rituximab
RCA	Regulators of complement activation (gene cluster)
r_s	Spearman correlation coefficient
SCR	Short consensus repeat
SD	Stable disease
Smad1	SMAD family member 1 (=Madh1)
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigens
Th1	T-helper 1
TIL	Tumor-infiltrating leukocyte
TNF	Tumor necrosis factor
TTF	Time to treatment failure
TUNEL	Tdt-mediated dUTP Nick-End Labeling
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

ABSTRACT

Rituximab (Mabthera®, Rituxan®) is a monoclonal antibody (mAb) against B-cell specific CD20 antigen. It is used for the treatment of B-cell non-Hodgkin lymphomas (NHL) and chronic lymphatic leukemia. In combination with chemotherapeutics rituximab is the first-line therapy of several types of NHLs including follicular lymphoma (FL). Immunochemotherapy has remarkably improved the outcome of NHL patients, but a vast variation in the lengths of remissions remains and the outcome of individual patients is difficult to predict. The cause of this variable behavior of tumors is poorly understood. This thesis has searched for an explanation for this issue by studying the effector mechanisms of rituximab and by comparing gene expression in lymphoma tissue samples of patients with long- and short-term survival.

Binding of rituximab to B-lymphoma cell activates cytotoxic cells and the complement (C) system, and induces apoptosis. In this work we have demonstrated that in cytoxicity assays C activation is the most efficient effector mechanism of rituximab. Incubation of rituximab-treated lymphoma cell lines with normal human serum (NHS) but not with peripheral blood leukocytes (PBL) resulted in significant cytolysis. Rituximab treatment induced apoptosis in cell-line dependent manner. These assays also showed that C-mediated cell killing could be markedly improved with simultaneous neutralization of the C regulatory proteins CD46 (Membrane cofactor protein (MCP)), CD55 (Decay-accelerating factor (DAF)), and CD59 (protectin) *in vitro*.

We studied the activation of the C system *in vivo* in blood and cerebrospinal fluid (CSF) before, during, and after intravenously administered rituximab. A marked increase in the concentration of the C activation marker C3a-desArg was measured in blood two hours after beginning of the first rituximab infusion. Emphasizing the role of C in mediating the action of rituximab a rapid disappearance of peripheral blood CD20 positive cells was observed simultaneously with C activation. Intravenously administered rituximab could not enter the central nervous system (CNS), and neither C activation nor removal of CD20 positive cells was observed in the CSF. This finding led to the suggestion that it might be worth of administering rituximab intrathecally to CNS-lymphoma patients.

Because *in vitro* data suggested that C regulators can affect the efficacy of rituximab, we conducted clinical studies where the differences in gene expression profiles of lymphoma tissue and in expression levels of C regulatory proteins were related to the outcome of 24 FL patients treated with immunochemotherapy. Expression of 18000 genes was analyzed from tumor sample isolated RNA using microarray technique. The patients were divided into "long-term responders" and "short-term responders", and the gene expression patterns between the groups were compared.

Lymphoma tissues of different patients were demonstrated to be genetically rather homogeneous, but the expression of certain genes was associated with different outcomes. The distinctive genes were often related to cell cycle regulation, signal transduction or regulation of immune responses. Of the best differentiating genes we randomly selected SMAD family member 1 (Smad1), macrophage receptor collagenous structure (Marco; a macrophage scavenger receptor), and Ephrin receptor A1 (EphA1; a tyrosine kinase involved in transendothelial migration) for further studies. The results were verified using quantitative polymerase chain reaction (qPCR) and immunohistochemical stainings. Smad1 and EphA1 were demonstrated to be mainly expressed in the microenvironment of the tumor cells. The staining intensity of EphA1 correlated to the clinical outcome in the study group of 24 patients and in a validation group of 40 patients. The results demonstrate that certain lymphoma tissue-associated factors correlate to the clinical outcome of immunochemotherapy treated FL patients. They also suggest that evaluation of these factors at the mRNA level using qPCR or at the protein level can be utilized to identify more specifically the patients for whom immunochemotherapy most probably is effective.

Low mRNA expressions of C regulators CD59 and especially of CD55 were also associated with a good prognosis of FL patients in the same data set. When lymphoma cell expressions were compared, progression free survival (PFS) times of patients with low CD55 or CD59 levels were significantly longer than of those with high expression levels. The results were verified using flow cytometry analysis in a prospective study of 12 patients. High expression of CD20 relative to CD55 correlated to a longer progression free survival. The difference between non-responding or early relapsed patients and patients in remission (after median follow-up of 22 months) became more obvious when CD20 expression was compared to combined average C regulator expression level. The results suggest that C regulatory proteins represent a clinically relevant immunotherapy escape mechanism of lymphoma cells. They also suggest that efficacy of immunotherapy and immunochemotherapy may be increased by neutralizing the C regulatory proteins.

In conclusion, we have demonstrated that activation of the C system is a clinically important effector mechanism of rituximab and that the tumor microenvironment and expression of C regulatory proteins affect markedly the efficacy of immunochemotherapy. This information can be used to identify more accurately patients to whom immunochemotherapy is beneficial. It may also help in the development of rituximab containing therapy, and other monoclonal antibody therapies.

1 GENERAL INTRODUCTION

The word cancer refers to a class of diseases characterized by uncontrolled division of cells and ability of the cells to invade and damage normal tissues either locally or at distant sites of the body. Cancers are rather common: 26000 people in Finland got cancer in 2004 and 11000 died of it (Finnish cancer registry 2005).

Human body consists of about 10^{14} cells, many of them dividing and undergoing programmed cell death (apoptosis) or dying of other reasons all the time. It has been calculated that about one million cells are produced and eradicated every second in our body. These million divisions are strictly regulated by hormones and growth promoting or suppressing signals, but all of them are vulnerable to errors, potentially leading to malignant transformation step by step. In this respect, we can ask: why aren't cancers far more common?

Fortunately, multicellular organisms have powerful surveillance mechanisms against potentially neoplastic cells: DNA replication errors are relatively rare and are actively repaired. Activation of many oncogenes, along with harmful effects, often activates apoptotic pathways, and intercellular signaling together with structures of tissue microenvironment usually restricts disseminated growth.

The immune system is a collection of specialized cells and substances that protect the body against potentially harmful invaders such as bacteria, viruses, fungi or parasites, but it can also recognize and eliminate endogenous objects such as immune complexes or transformed cells. An essential prerequisite for the development of cancer is that the tumor cells can evade immune elimination. If they succeed in this, cancer may be the result. Even if immune system by itself is not capable of preventing the growth of malignancies in these situations, the progress of medical sciences has made it possible for the body to strike back once more. Various immunotherapeutic approaches can strengthen the already existing but weak anti-tumor activity or generate an immune system attack against specifically defined targets.

An intentional exploitation of the immune system for cancer treatment dates back to the early 20th century, when William B. Coley, a surgeon in New York City, discovered that malignant tumors disappeared in cancer patients who had contracted streptococcal infections. He thereafter deliberately infected patients carrying non-treatable malignancies with killed bacteria, and actually created the first anti-cancer vaccine. *Coley's mixed bacterial toxin* resulted in the regression of tumors in one third of the patients. Unfortunately, his results were unpredictable, and were slowly forgotten for decades. The science of immunology was very young on those days, and his observations could not be mechanistically explained. At the other side of the Atlantic Ocean, also during the late 19th and early 20th century Paul Ehrlich dreamed of "magic bullet". Using our terminology

he proposed that cells have specific receptors for antigens and that they can shed these receptors into the blood when they come into contact with antigen. This was the first time when the nature of antibodies was suggested.

From those days it took almost a century before the first specific immunotherapy against cancer received acceptance for clinical use. This "magic bullet" was rituximab, a monoclonal antibody against the B-cell specific CD20 antigen, and it was launched for the treatment of non-Hodgkin lymphoma. Simultaneously, a whole array of new unanswered questions emerged for both basic scientists and clinicians to address. What happens when an anti-tumor antibody recognizes its target? Why don't all CD20 positive cells die, even if they have been recognized by rituximab? Why don't all patients respond to rituximab? Why the lengths of remissions differ so much between individual patients even if their primary responses had seemed to be very similar? –The aim of this study was to investigate these interesting questions.

2 REVIEW OF LITERATURE

2.1 IMMUNE SYSTEM

The immune system is responsible for protecting the host against potentially harmful agents. The immune system has traditionally been divided into the innate and adaptive (specific) parts (Riot, Brostoff & Male 1996). Innate immunity refers to pre-existing immediate defense mechanisms that the host uses against invading micro organisms and for clearance of debris. Adaptive immune system responses take weeks to develop, but the reactions are powerful and aimed accurately against specific targets.

INNATE IMMUNITY

Existing already in ancient organisms, the innate immune system is evolutionary old but has developed over millions of years to become a clever, complex entity of different mechanisms. In humans, innate immunity includes mechanical, chemical, humoral and cellular defense networks. When a microbe is about to invade the body, it first faces the physical barriers such as skin or mucosal membranes (Male, Roitt 1996). Cilia of the respiratory tract transfer invading microbes away from the body; mucus traps pathogens trying to breach the mucosal barriers; lysozyme of tears and saliva destroys bacteria etc. However, if a microbe has succeeded to invade the body, the cellular and humoral parts of the innate immune system are immediately alerted and mobilized. The reactions of innate immunity are fast, because, instead of mounting a response against distinct foreign antigens, the innate immune system can identify common structures present in many microorganisms. These structures include lipopolysaccarides of gram-negative bacteria, bacterial peptidoglycans and viral double-stranded RNA. Cells of the innate immune system include neutrophils, macrophages, monocytes, dendritic cells, eosinophils, basophils, mast cells and natural killer (NK) cells. The complement system is an essential part of humoral innate immunity.

ADAPTIVE IMMUNITY

Adaptive immune system is evolutionary much more novel than the innate immune system and found only in multicellular organisms. Adaptive immune system is organized through two classes of specialized cells, T and B lymphocytes, that can recognize a wide variety of different antigens (Male, Roitt 1996). Reactions of adaptive immune system are extremely powerful and specific, but they do not usually occur before antigen-presenting cells (APC) have recognized invading agents and processed them for presentation to T cells. The reactions take one to two weeks to develop, but simultaneously develops a long-lasting memory of how to combat efficiently these specific microorganisms.

ANTIBODIES

An antibody is a soluble or B-cell membrane-associated (B-cell receptor) molecule used by the immune system to identify specifically foreign objects like bacteria and viruses. An antibody can recognize and bind to antigens on target cell surfaces or in solution. This

binding can lead e.g. to direct neutralization of a virus, blocking of the function of antigen or labeling of the target for attack by other parts of the immune system (Turner 1996).

Antibodies are Y-shaped glycoproteins formed by two identical heavy chains and two identical light chains (Figure 1). Based on different heavy chain constant domains antibodies are divided into five types with different biological properties and locations: Immunoglobulin (Ig) A, IgD, IgE, IgG, and IgM.

Fab-regions at the "two arms" of the antibody contain the antigen-binding sites and define the specificity of the antibody (Figure 1). Fc-region mediates the physiologic effects of the antibody by binding the complement component C1q and to Fc-receptors of various cells. Different immunoglobulin classes have different abilities to trigger complement. Best activators of C system are subclasses IgG1 and IgG3. By binding to different leukocyte Fc-receptors antibodies can trigger other effector mechanisms such as phagocytosis, antibody-dependent cellular cytotoxicity and acute inflammatory responses.

The recognition of numerous specific antigens requires a large number of different antibodies. To enable the generation of required antibody repertoire, specific mechanisms for their production in B-cells are needed. Availability of multiple V, D and J genes, their somatic recombinations and somatic hypermutations allow the production of much larger diversity of antibodies than could be expected based on the availability of different genes alone (Hay 1996).

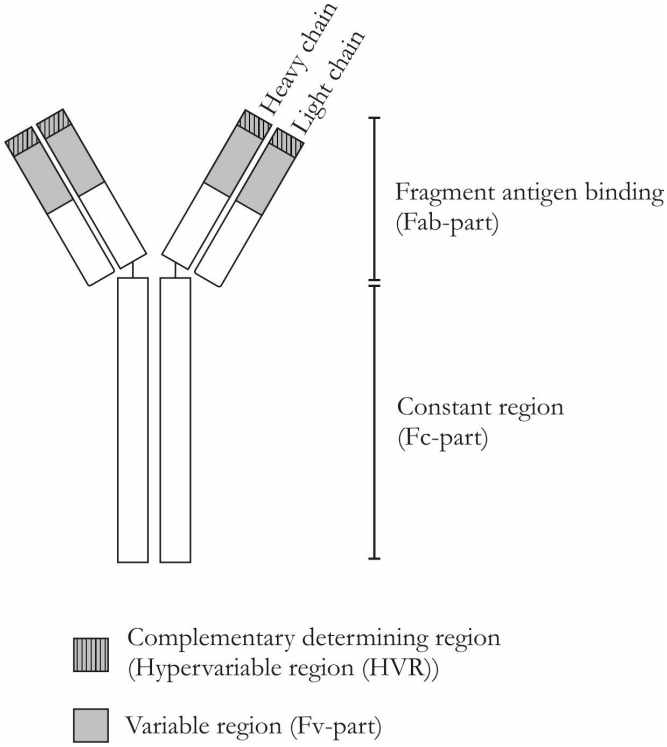

Figure 1. Basic structure of an antibody

2.2 COMPLEMENT SYSTEM

OVERVIEW AND PHYSIOLOGY

The complement system forms an essential part of both the innate and adaptive immune systems. It is a group of plasma proteins that interact in a cascade-like manner. C system consists of over 30 soluble and membrane-bound proteins, of which most function as enzymes, binding proteins, receptors or regulators of activation. The main physiological functions of complement are (1) protection against infections, (2) generation of inflammation, (3) bridging between innate and adaptive immunity, and (4) clearance of immune complexes, damaged cells or other debris (Walport 2001).

When C faces a foreign microbe, it becomes activated and the cascade can prodeed to generation of membrane attack complexes (MAC) and direct cytolysis. C can also promote phagocytosis by opsonizing foreign particles with its components C1q, iC3b, C3b and C4b, and attract and activate leukocytes via releasing chemotactic and anaphylatoxic agents C5a, C3a.

Target recognition by C occurs often non-specifically and C operates as a part of innate immunity. On the other hand, antigen- or immunecomplex bound C3b and C4b molecules can activate B cells, follicular dendritic cells or other antigen-presenting cells by binding to the CR1 (CD35) and CR2 (CD21) complement receptors, and induce adaptive immune system reactions. Also, if antibodies have already been generated and they have recognized their targets, the C system can become activated and direct C mediated cytolysis can take place.

C ACTIVATION PATHWAYS

The C system can become activated through three distinct routes (Figure 2) (Walport 2001). (I) The classical pathway (CP) usually becomes activated by antibodies. It mediates specific immune responses and functions as a part of adaptive immunity. (II) The alternative pathway (AP) forms a major part of the humoral immune system's natural defense against infections. It operates continuously at a slow rate in plasma, and becomes activated abundantly wherever foreign surfaces are present. The alternative C pathway is an important discriminator between self and non-self structures and can amplify C activation reactions initiated via other pathways. (III) The lectin pathway (LP) also functions as a part of innate immunity by recognizing mannan and N-acetylglycosamine residues on microbial surfaces.

Classical pathway

When antibodies bind to their target molecules, they can initiate the CP of complement. The CP is triggered when the complement molecule C1q binds to the Fc-parts of antigen-bound IgM- or IgG-class immunoglobulins. The CP can also be activated antigen-independently by C-reactive protein (Miyazawa, Inoue 1990) or by surface blebs of apoptotic cells (Navratil et al. 2001). The binding to antibody causes conformational

changes in the C1q molecule leading to activation of two C1r proteases and further to cleavage of two C1s (another serine protease) molecules. The C1-complex now binds to and splits C2 and C4. The cleavage product C4b binds covalently to a cell surface and forms C4bC2 complexes. The activated C1s further cleaves C4bC2 and generates C4bC2a, which is the classical pathway C3/C5 convertase.

Alternative pathway

In the fluid phase spontaneous hydrolysis of C3 to C3(H_2O) occurs continuously at a low rate (Pangburn, Muller-Eberhard 1983). When C3(H_2O) binds to factor B, and factor D cleaves factor B to Bb, C3(H_2O)Bb convertase is generated. This convertase will cleave C3 to C3a and C3b, of which the latter can bind to microbial surfaces. The cascade is preceded, when C3b binds to factor B and is cleaved to C3bBb by factor D. The C3bBb complex is the principal AP C3 convertase, which will produce more C3b and C3a. On foreign surfaces the lack of C regulatory molecules leads to deposition of C3b molecules and continuing activation of the alternative complement pathway (Pangburn, Muller-Eberhard 1984). In this way the target surface will be covered by numerous C3b molecules. When C3bBb recruits another C3b molecule, a C5 convertase is generated.

Lectin pathway

The lectin pathway is homologous to the classical pathway. This pathway is activated when mannan-binding lectin (MBL) binds to sugar structures on target surfaces (Petersen et al. 2000). MBL-associated serine proteases (MASPs) 1 and 2 bind to target-bound MBL and form a complex that can cleave C4 and C2. This generates the classical pathway C3-convertase, C4b2a (Figure 2).

Terminal complement pathway

All C activation pathways lead to the formation of C3/C5 convertases and cleavage of numerous C3 and C5 molecules. The generated C3b molecules cover the target cell surfaces favoring phagocytosis and other biological responses, and C5b molecules initiate the terminal complement pathway.

The terminal complement pathway consists of factors C5, C6, C7, C8 and C9. C5b molecules can bind in the fluid phase C6 and C7 and on membranes C8. The C5b-8 complex catalyzes polymerization of multiple C9 molecules to form a pore-like structure on the target cell membranes (Malinski, Nelsestuen 1989). The generation of membrane channels, MACs, leads to calcium-influx, leakage of several intracellular substances, loss of mitochondrial membrane potential, and lysis of the target cell.

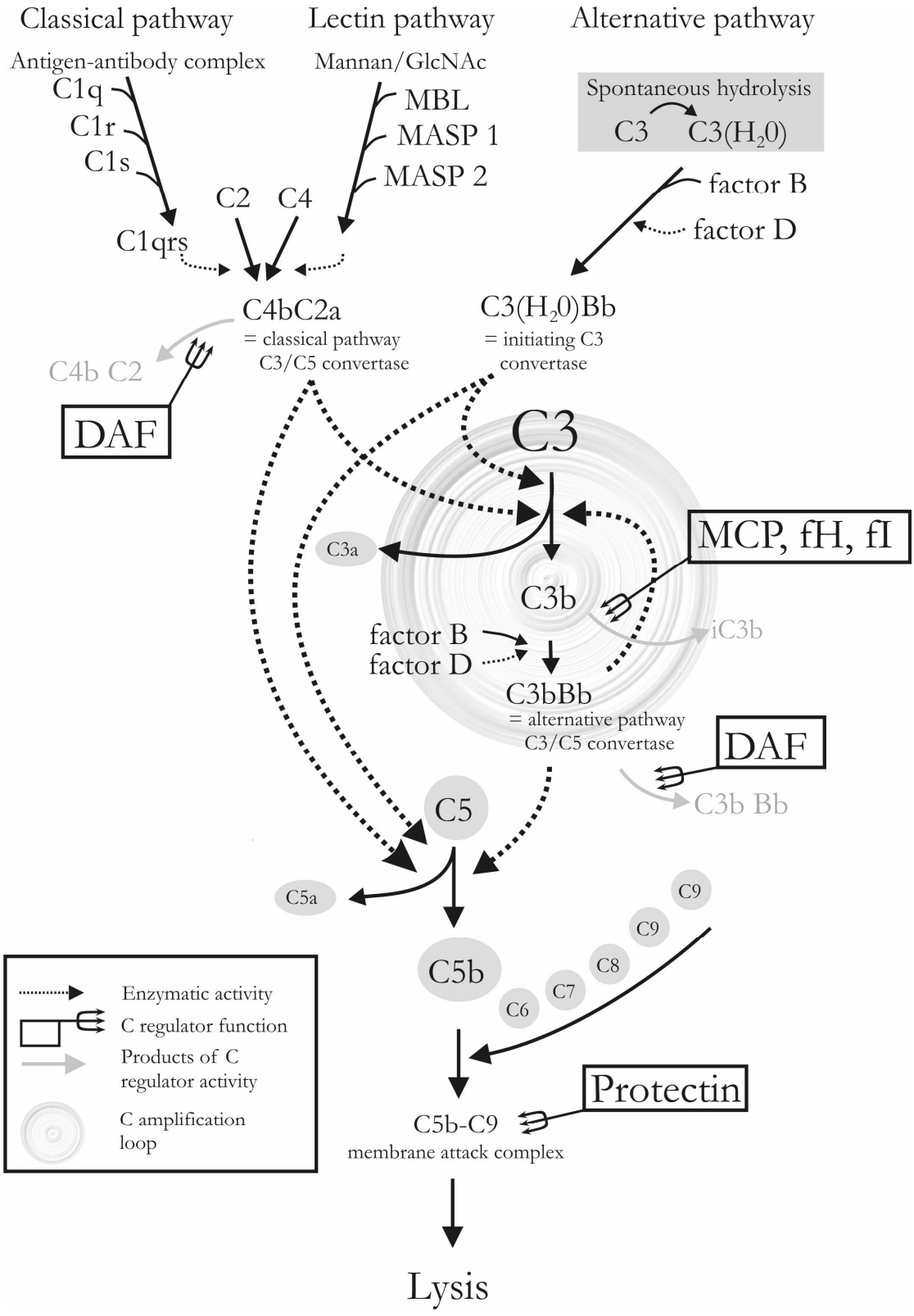

Figure 2. Complement activation pathways

REGULATION OF COMPLEMENT ACTIVATION

Complement activation must be strictly controlled because C-induced inflammation and tissue destruction can be harmful to the host. Inappropriate C activation, excessive inflammation and overconsumption of C components are avoided in normal tissues mainly by the expression of complement regulatory proteins. The distribution and importance of different membrane-bound and soluble C regulators vary between tissues and cell types (Gorter, Meri 1999, Gorter, Meri 1999, Morgan, Meri 1994). Of the membrane bound regulators CD46 (Nicholson-Weller et al. 1982, Nicholson-Weller et al. 1985), CD55 (Seya, Turner & Atkinson 1986, Sugita, Nakano & Tomita 1988), CD59 (Davies et al. 1989, Holguin et al. 1989, Meri et al. 1990), and of the soluble regulators factor H (Fontaine, Daveau & Gilbert 1983) and C4 binding protein (C4bp) are best characterized and considered as most important. The major C regulators are listed in Table 1. The different regulators affect the progress of C activation at different phases, and therefore their actions are often synergistic. In addition to C regulators also other C regulation mechanisms exist. E.g. MAC complexes can be eliminated from plasma membranes by endocytosis and by vesiculation (Campbell, Morgan 1985, Scolding et al. 1989, Sims, Wiedmer 1986).

Table 1. A list of most important soluble and membrane-bound C regulators.

Soluble regulators	*Main function*
C1-inhibitor	Binds and inactivates C1r and C1s
C4-binding protein	Inhibits formation and inactivates classical pathway C3 convertase
Factor H	Inhibits formation and inactivates alternative pathway C3 convertase
Factor I	Cleaves and inactivates C3b and C4b
Vitronectin	Inhibits binding of terminal complement complex to cell membranes
Clusterin	Inhibits binding of terminal complement complex to cell membranes
Membrane-bound regulators	*Main function*
CD35 (Complement receptor 1)	Dissociates alternative and classical pathway C3/C5 convertases, cofactor for factor I
CD46 (MCP)	Cofactor for factor I
CD55 (DAF)	Dissociates alternative and classical pathway C3/C5 convertases
CD59 (Protectin)	Prevents formation of membrane attack complexes

CD46

CD46 or the membrane cofactor protein is a 51-68 kDa glycoprotein consisting of four extracellular short consensus repeat (SCR) domains, a membrane-proximal region, a transmembrane domain, and an intracellular cytoplasmic tail. The gene for MCP is located in chromosome 1. CD46 gene forms a "regulators of complement activation" (RCA) gene cluster (1q32) together with genes of other C regulators with SCR domains (Rodriguez de Cordoba et al. 1985). Probably generated by alternative splicing and glycosylation, CD46 can be present on the same cells in different isoforms (Post et al. 1991). The biological meaning of this is not known.

CD46 is expressed by almost all nucleated cells, including leukocytes, but is not present in erythrocytes (Seya et al. 1988). It acts as a cofactor for factor I and thus promotes cleavage of C3b to iC3b, and of C4b to C4c and C4d. Thereby CD46 functions as an inhibitor of CP and especially of the AP C3/C5 convertases (Kojima et al. 1993).

CD55

CD55 or decay-accelerating factor is a 190-220 kDa membrane glycoprotein. It also belongs to the RCA-group. CD55 has four SCR domains and a glycosyl-phosphatidyl-inositol- (GPI-) anchor that links it to membrane phospholipids (Caras et al. 1987, Medof et al. 1986). CD55 is distributed widely in different tissues, but usually less abundantly than CD46. It is also expressed in erythrocytes, but is absent from some T cells and NK-cells (Nicholson-Weller, Russian & Austen 1986, Tomita, Okada & Okada 1991). In addition to GPI-anchored form also a soluble form of CD55 lacking the GPI-anchorlipid is found in serum and other body fluids (Medof et al. 1987).

The main function of CD55 is to inhibit C activation at the C3 level by accelerating the decay of both CP and AP C3/C5 convertases (C4b2a and C3bBb). Besides the C inhibiting function, CD55 has also been shown to promote tumorigenesis (Loberg et al. 2006) and to inhibit function and migration of NK and T cells (Kusama et al. 2003, Mikesch et al. 2006, Spendlove et al. 2006).

CD59

CD59 or protectin is a 18-23 kDa GPI-anchored membrane glycoprotein. Not consisting of SCRs but of another distinct domain, it differs structurally from CD46 and CD55. The *CD59* gene is located in chromosome 11p13. CD59 was first isolated from human erythrocytes, but has later been found on all circulating cells and widely also on endothelial cells, and epithelial cells (Davies et al. 1989, Meri, Waldmann & Lachmann 1991). In addition to the GPI-lipid anchored form, there is a soluble form of CD59, which has been detected in various body fluids including urine, tears, and saliva. CD59 represents the last line of defense against C activation. It can bind to C8 or C9 and prevent polymerization of C9 and MAC formation (Meri et al. 1990).

2.3 CANCER IMMUNOLOGY

The immune system is much better in recognizing foreign organisms than body's own transformed or mutated cells. Nevertheless, studies of the immune system have revealed that the human body defends itself against cancer principally in much the same way as it does when eliminating invading microbes.

In the late 1950s Frank MacFarlane Burnet (Burnet 1970) and Lewis Thomas (Thomas 1982) separately launched the theory of immunosurveillance. They hypothesized that the immune system is continually surveying for abnormal cells. Precancerous and cancerous cells are in most cases recognized as non-self structures, and destroyed. Along their theory, it is a rare phenomenon that sufficient amounts of tumor cells manage to escape the immune system, but if they do, cancer is the result. The observations that transplantation of tumor tissue between syngeneic animals lead to rejection, but that transplantation of normal tissue does not, revealed the existence of tumor-associated antigens (TAA). Later the theory was criticized from several directions. In 1970s, differences in tumorigenesis between immunocompetent and partly immunodeficient mice were not observed (Stutman 1974, Stutman 1979) and the immunosurveillance theory was almost abandoned. Many oncologists and scientists believed that instead of immune system other innate fail safe-mechanisms prevent tumors from arising that often. They thought that the immune system usually cannot recognize precancerous cells as foreign and therefore cannot have such a central role in tumor elimination.

There were, however, certain epidemiological and other observations, that suggested that immune system might still have some impact. (I) The incidence of some malignancies (e.g. Kaposi's sarcoma and immunoproliferative diseases) is increased in immunodeficient patients (Boshoff, Weiss 2001). (II) Cancer more often develops in young children or senior citizens – groups of people also having weaker immune defenses (Finnish cancer registry 2005). (III) Certain tumors sometimes regress spontaneously (e.g. melanoma, renal cell carcinoma) (Papac 1996) – it has been speculated that only immune reactions can explain these "miraculous" healings. (IV) Anti-tumor antibodies and immune T-lymphocytes have been detected in cancer patients (Bremers, Parmiani 2000). These observations suggest that immune responses develop against some types of tumors, particularly against those, where viruses play a role in tumor development.

New studies in mice in the 1990s and early 21st century have brought back the ideas of immunosurveillance. They have demonstrated that mice really lacking an intact immune system generate more spontaneous and chemically-induced tumors than immunocompetent mice. In addition, tumors from immunodeficient mice are more immunogenic than those from wild type mice. Broadly based on these observations Dunn and his colleagues (Dunn, Koebel & Schreiber 2006, Dunn, Old & Schreiber 2004a, Dunn, Old & Schreiber 2004b) have proposed a theory of *cancer immunoediting*, in

which the interaction between a developing tumor and host immune system is comprised of three phases. *The elimination* phase resembles the actions described within the immunosurveillance theory. The innate and adaptive arms of the immune system can recognize and kill most of the transformed cells, and reject the tumor. If the elimination is incomplete and some malignant cells survive, immunoediting proceeds to an *equilibrium* phase. In the equilibrium phase residual cancer cells persist, but an immune pressure prevents them from expanding and generating a clinical disease. This stage can last for years, and the disease can stay in clinical remission. But if the balance is once lost and the tumor cells start growing progressively, the process proceeds to an *escape* phase. The escape can occur, if new (e.g. oncogenic) mutations take place, if tumors develop new immune escape mechanisms, or if the immune system is exhausted. In this situation the immune system cannot control the tumor growth, and a relapse or wide-spread disease results.

Another question is how are malignant cells recognized in the first place? Most scientists believe that tumors express "non-self" antigens, i.e. newly mutated antigens or antigens expressed only at low levels by normal cells. If such antigens are detected by the immune system, an immune response also needs a secondary signal to be generated efficiently. The innate immune system can provide the secondary stimulation through up-regulation of co-stimulatory molecules in APCs (Lafferty, Cunningham 1975). According to the self/non-self discrimination theory, APCs can trigger the adaptive immune system after recognizing conserved molecular patterns usually associated with evolutionary distant structures such as microbial surfaces. And it is the lack of this signal that in the case of cancer prevents an immune response to take place.

According to another theory, the so called *danger model,* the immune system is stimulated by endogenous signals that originate from stressed or injured cells (Fuchs, Matzinger 1996, Matzinger 1998). For instance, an abnormal damage of cells is a signal for local APCs to takeup local antigens and to upregulate the costimulatory molecules needed for activation of T cells. During metastasis and invasion of malignant cells, natural tissue barriers are disrupted, and proinflammatory signals are released. This can induce an immune response against these cells without the discrimination between self and non-self.

COMPLEMENT AND CANCER

Limited information is available about the tumor immunosurveillance function of C. In principle the C system has the ability to destroy tumor cells, but compared to cell-mediated adaptive immunity it has much less discriminating power between self and foreign. No clear evidence that C would have a clinically important role in protection against cancer, nor associations between C deficiencies and an increased cancer incidence, has been published. However, various C abnormalities such as elevated plasma C3 levels in brain tumor patients (Matsutani et al. 1984) and lung cancer patients and reduced complement titers in breast, gastric and colon–rectum carcinoma patients

(Mangano et al. 1984) have been described. Also C deposits within the tumor tissue, especially in necrotic areas, have been found in e.g. breast (Niculescu et al. 1992), papillary thyroid (Lucas et al. 1996), and renal (our unpublished data) carcinomas. Furthermore, Varga and her colleagues demonstrated that low pre-treatment classical pathway C levels correlated unfavorably to long-term prognosis of chronic lymphatic leukemia (CLL) patients (Varga et al. 1995). None of these observations, however, provides evidence that tumor cells by themselves could activate the C system *in vivo*, or that the C system could affect the growth or development of tumors. It is possible that the C system becomes activated in the tumor tissue indirectly by immune complexes, necrotic cells or substances generated by tumors. In the studies by Varga infections were also more common in patients with low C activity. Therefore it was hypothesized that it may also be the increased susceptibility to infections that explains the unfavorable prognosis of CLL patients with low classical pathway C levels.

C RESISTANCE MECHANISMS OF TUMORS

Even if the C system would not be the major factor responsible for tumor growth restriction, it is fundamental for malignant cells to have mechanisms to resist C attack *in vivo*. Several different C escape mechanisms of tumors have been found or proposed. Most importantly, malignant cells are protected against C by membrane-associated and soluble C regulatory proteins. Other less thoroughly characterized C escape mechanisms include membrane expression of ecto-proteinases, which can control C3 deposition (Jean et al. 1995, Jean et al. 1996, Ollert et al. 1990), or kinases that can phosphorylate C9 and affect MAC formation (Paas, Bohana-Kashtan & Fishelson 1999). Tumor cells can also secret proteochondroitin sulfate, which inhibits formation of the C1 complex (Kirschfink et al. 1997). It has also been demonstrated that sublytic amounts of MAC cause various biological effects including an increased resistance to C lysis. This resistance has been associated with an increase in intracellular Ca^{2+} levels, and activation of cytoplasmic protein kinase C (PKC), MAP kinase (MAPK) and protein synthesis (Fishelson, Kraus 1998). In addition, shedding of C3b-bound surface molecules or formation of MAC-containing vesicles may provide protection against C attack (Whitlow, Klein 1997).

Expression of membrane bound and soluble C regulators in tumors

All hematological malignancies and the majority of solid tumors express at least some of membrane bound C regulators, that include CD46 (MCP), CD55 (DAF), and CD59 (protectin). The expression levels of C regulators in malignant tumors are usually equal to, or greater, than seen in the surrounding normal tissue (Bjørge et al. 1997, Hakulinen, Meri 1994, Hofman et al. 1994, Jarvis et al. 1997). Membrane expression of C regulators has been demonstrated e.g. in glioma (Junnikkala et al. 2000, Mäenpää et al. 1996), carcinomas of thyroid gland (Yamakawa et al. 1994), breast (Hakulinen, Meri 1994, Hofman et al. 1994), ovary (Bjørge et al. 1997), lung (Niehans et al. 1996) cervix (Simpson et al. 1997), colon (Koretz et al. 1992, Koretz et al. 1993), and kidney (Niehans et al. 1996) and in leukemias and lymphomas (Fukuda et al. 1991, Hara et al. 1992, Seya et al. 1994). Interestingly, deficiencies in C regulators have also been reported in a subset

of breast cancers (Hofman et al. 1994), and some NHLs were reported to lack CD55 expression (Fukuda et al. 1991, Hara et al. 1992, Seya et al. 1994).

Soluble forms of CD46, CD55 and CD59 have been found in various body fluids. Release of these soluble forms can either augment the local complement inhibitory potential of tumors or reduce the C regulatory function of tumor cells. Hakulinen et al. demonstrated that shedding of CD46 from cell membranes is regulated by a disintegrin and metalloproteinase (ADAM) 10 (Hakulinen, Keski-Oja 2006). ADAM10-mediated release of CD46 was stimulated e.g. during apoptosis, and led to reduction of C regulation capacity of these cells.

Factor H (fH) and other mainly soluble C regulators are also involved in tumor cell resistance against C (Junnikkala et al. 2002). Certain tumor cells have been shown to bind fH molecules from circulation and in that way increase their C resistance (Junnikkala et al. 2002).

External modulation of C regulator expression

Expression levels of C regulators may be influenced by host-derived and microenvironmental factors such as hormones, growth factors, and cytokines. For example interferon-γ (IFNγ) has been shown to increase CD59 and CD55 expression in gastrointestinal tumor cells (Schmitt et al. 1999) and interleukin-1β (IL-1β) has been found to upregulate CD46 expression in colon carcinoma (Bjørge et al. 1995). Tumor necrosis factor-α (TNFα), IL-1β, and IL-6 have been demonstrated to increase the expression of CD55 and CD59, but to decrease the expression of CD46 in hepatoma cells (Spiller et al. 2000). Also, hypoxemia has been reported to reduce the expression of CD59 (Väkevä, Meri 1998). On the other hand in colorectal cancer it has been reported that hypoxia induces COX-2 expression and increases prostaglandin E2 levels, which further upregulates the expression of the complement inhibitor CD55 (Holla et al. 2005, Liu et al. 1999). Undoubtedly information about the modulation capacity of cytokines and the effect of oxygen supply are limited, and based on these individual findings it is difficult to evaluate the clinical importance of the phenomenon.

2.4 IMMUNOTHERAPY

Cancer immunotherapy aims at exploiting the therapeutic potential of tumor-specific antibodies and humoral or cellular immune effector mechanisms. In active immunotherapy an endogenous, long-lasting immune response is induced, whereas in passive immunotherapy ex vivo produced immune system components such as antibodies are used. Besides this division different immunotherapeutic methods can also be divided into specific and non-specific immunotherapies. In non-specific immunotherapies existing or naturally-induced immune reactions are strengthened by humoral or cellular factors. In specific immunotherapies the target cells are attacked with antibodies, vaccines or cells prepared specifically against desired targets.

NON-SPECIFIC IMMUNOTHERAPIES

Cytokines

Cytokines can affect the growth of tumor cells either directly or via activating cells of innate or adaptive immune system. As examples of direct effects TNF-α, IFN-α and β, and IL-4 and 6 can induce tumor cells to commit suicide or to stop further growth. IFN-α has been studied for the treatment of metastatic melanoma, renal cell carcinoma and follicular lymphoma (Rohatiner et al. 2005).

Clinically more important applications have involved indirect effects of cytokines. Most importantly, IL-2 has been observed to strengthen anti-tumor immune responses via promoting the growth and activation of T-cells and NK cells. IL-2 has been used in the treatment of metastatic melanoma and renal cell carcinoma, often together with chemotherapeutics. IFN-α2b has been approved for the treatment of Kaposi's sarcoma and certain subtypes of leukemia (Hurley, Chapman 2005, Yang et al. 2003). Often side effects have been found to be a problem in cytokine treatments, but combinations of different cytokines have had synergistic effects with fewer side effects.

Lymphokine-activated killer (LAK) cell and tumor-infiltrating lymphocyte (TIL) therapies

Human peripheral blood mononuclear cells (PBMCs) can be isolated, treated *ex vivo* with IL-2 to generate lymphokine-activated killer (LAK) cells, and then re-injected to the patients. Sometimes even complete tumor regressions have been observed (Rosenberg et al. 1993). After initially promising results clinical studies have later failed to show enough efficacies (Weber et al. 1992).

To improve the results of immunotherapies new strategies to select more accurately the anti-tumor-lymphocytes have been proposed. In tumor-infiltrating lymphocyte (TIL) therapy lymphocytes are collected from inside of surgically removed tumor tissue, grown in laboratory conditions, treated with IL-2, and injected back to the patient. TILs have being tested for example in clinical trials of melanoma and renal cell carcinoma (Dudley et al. 2002, Dudley et al. 2005, Rosenberg, Spiess & Lafreniere 1986), but the responses have been only moderate. Probably because the production of TILs is technically difficult randomized trials are still missing (June 2007).

SPECIFIC IMMUNOTHERAPIES

The T and B cell based components of the immune system can induce specific immune responses against tumors.

Adoptive transfer of T cells

In the so called adoptive transfer, tumor antigen-specific T cells are selected and isolated from cancer patients. Analogously to the above mentioned LAK and TIL therapies the cells are expanded in test-tubes and re-infused back to the patient to kill the remaining

tumor cells. Clinical studies have shown promising results in metastatic melanoma (Morgan et al. 2006).

Vaccination

Cancer vaccines are cells, parts of cells, antigens (individual or mixed) or DNAs, which are injected into the body to trigger hosts' own immune system to respond against tumors. The mounted reactions are not generalized but specific, affecting only the cancer cells. Successful cancer vaccines can induce the immune system to make antibodies or killer T cells against specific anti-tumor antigens. To strengthen the immune responses, vaccines may be combined with adjuvants.

Tumor cell vaccines. Tumor cell vaccines are prepared after surgery of the primary tumors. Malignant cells from tumors are separated, killed and re-injected into the patient to stimulate a specific immune response against any similar cancer cell remaining in the body. These vaccines are autologous, but tumor cell vaccines can also be allogeneic. In this case the cancer cells, usually removed from several donors, are used along with adjuvants to stimulate the immune system.

Dendritic cell vaccines. T cells can only recognize antigens that have been processed and presented to them by APCs. Therefore, dendritic cells (DCs) and other APCs are potentially suitable tools as vaccines against cancer. DCs are first isolated from the patient, and then exposed to tumor antigens (also derived from patients) *ex vivo*. The transfer of these antigen-loaded DCs back to the patient can induce significant tumor-specific immune responses. The technique has been demonstrated to be effective in some smaller clinical trials (Banchereau et al. 2001, Yu et al. 2001), but failed to show effect in an important phase III melanoma trial (Gilboa 2007, Schadendorf et al. 2006).

Protein- and peptide vaccines. In antigen vaccines the immune system is stimulated by specifically selected antigens instead of whole tumor cells. In peptide vaccines the tumor antigens are processed to resemble epitopes presented by major histocompatibility complex (MHC)-molecules and injected to the patients together with appropriate co-stimulatory molecules. The main advantage of peptide-based vaccines is that they are not patient-specific and they can raise immune responses against particular antigens without a risk of infusing dangerous substances into the patient.

Anti-idiotype vaccines. B-cell malignancies arise from a single B-lymphocyte clone and therefore all lymphoma cells carry the same unique immunoglobulin on their surfaces. These immunoglobulins can be used as targets for vaccination. The challenge is that a customized vaccine should be prepared for each patient. New technologies, in which the idiotype genes are amplified and cloned by PCR, and then transfected to cell cultures, have provided better possibilities for production of idiotype vaccines. In recently published clinical study idiotypic vaccination induced specific immune responses with clinical benefit for follicular lymphoma patients (Inoges et al. 2006, de Cerio et al. 2007).

DNA vaccines. DNA vaccines are bacterial plasmids including DNA that codes for desired antigens. Injected DNA makes the body to produce itself the proteins against which an immune response will develop. DNA vaccines can be designed to induce different effector pathways against tumor targets. They may e.g. induce production of antibodies against cell-surface antigens or activate cytotoxic T lymphocytes to respond against intracellular antigens expressed only as MHC class I-associated peptides. To amplify anti-tumor immune responses genes encoding foreign proteins may be fused to tumor-derived sequences. DNA fusion gene vaccines have been designed against B-cell malignancies by fusing idiotype-related single-chain Fv sequence to tetanus toxin-derived fragment C (King et al. 1998, Spellerberg et al. 1997). Pivotal clinical trial in follicular lymphoma showed promising results (Stevenson et al. 2004).

Monoclonal antibodies

Monoclonal antibody therapy is passive immunotherapy, in which antibodies against tumor-specific antigens are produced in the laboratory and infused into the patients. The target antigens are molecules selectively or abundantly expressed in the tumor cells or in the type of cells from which the tumor is derived. To achieve a clinical effect, large amounts of antibodies are needed, but the hybridoma and cell culture techniques provide possibilities for large scale antibody production.

When a suitable candidate antigen for immunotherapy has been found, monoclonal antibodies can be developed by immunizing mice with it. Mouse B-cells are harvested after immunization and fused with a mouse myeloma cells to produce hybridomas. The unique cell that produces antibodies against the desired target is selected. When this single hybridoma cell is multiplied, numerous identical hybridoma cells producing identical antibodies, monoclonal antibodies are obtained (Kohler, Milstein 1975). Genetic engineering, like the development of strong promoters, has increased the productivity of these cultures, and nowadays sufficient amounts of antibodies for commercial use can be produced.

Monoclonal antibodies are consumed upon binding to target antigens and have a limited life-span. Therefore repeated infusions to the patients are needed. However, repeated application of foreign antibodies to humans poses a threat of inducing antibody responses against the injected antibodies themselves (Frodin, Lefvert & Mellstedt 1992). The risk has been diminished by developing chimeric and humanized antibodies. Chimeric antibodies are constructed by combining the mouse antibody-derived Fab part with human antibody Fc-region, whereas in humanized antibodies only the six antigen-binding polypeptide loops are of non-human origin.

The biologic effects of monoclonal antibodies are multiple. They may crosslink the target antigens, neutralize soluble signaling proteins, block receptor binding sites or induce apoptotic pathways. E.g. trastuzumab (Herceptin) can inhibit the growth of breast cancer cells by binding to human epidermal growth factor receptor 2 (HER2) (Hudis 2007).

HER2 signaling promotes cell proliferation and trastuzumab binding has been suggested to decrease HER2 signaling by preventing HER2-receptor dimerization, by inhibiting shedding of extracellular domain of HER2 receptor or by promoting endocytotic destruction of the receptor. Some models have also suggested that trastuzumab can recruit immune effector cells.

Therapeutically one of the most important functions of monoclonal antibodies is indeed the ability to activate leukocytes or the complement system. Immunoglobulins of different classes have different abilities to activate complement and Fc-receptor carrying cytotoxic cells. The most potent C activating antibodies are IgG subclasses IgG1 and IgG3 (Wallace, Howell & Fanger 1994). Therefore, antibodies of these types are most commonly used as immunotherapeutic agents. The mechanisms whereby antibodies can mediate target cell damage are multiple (Figure 3). In complement dependent cytotoxicity (CDC) antibodies activate the C system, and if C activation is not restricted, it proceeds to formation of MAC and lysis of the target cell. In antibody-dependent cellular cytotoxicity (ADCC) monocytes, macrophages, NK-cells and other cytotoxic cells can recognize Fc-parts of tumor-bound antibodies with their Fc-receptors become activated and kill the targets. The complement system and cellular mechanisms can also act synergistically. In complement-dependent cellular cytotoxicity (CDCC) cell-mediated cytotoxicity or phagocytosis is facilitated, when phagocytes or cytotoxic cells recognize deposited C components C1q, C3b, inactivated C3b, and/or C4b. Moreover, the immune reactions are further enhanced, because C factors C3a and C5a are chemotactic and cell-activating anaphylatoxins.

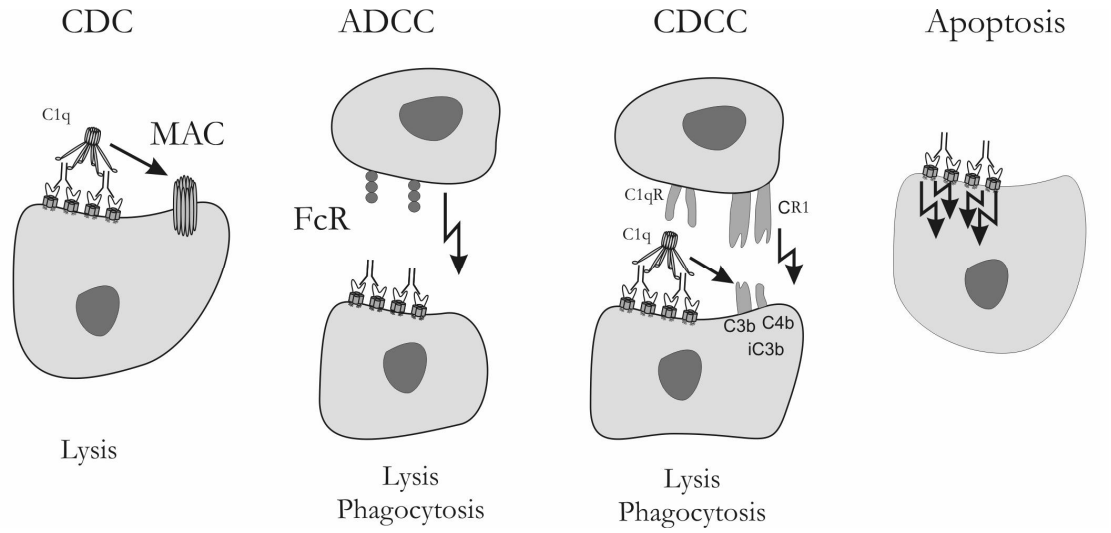

Figure 3. The major effector mechanisms of antibodies.
CDC = Complement dependent cytotoxicity, ADCC = Antibody-dependent cellular cytotoxicity, CDCC = Complement dependent cellular cytotoxicity, MAC = Membrane attack complex, FcR = Fc-receptor, CR1 = Complement receptor 1 and C1qR = C1q-receptor)

The technique of monoclonal antibodies was developed in the 1960s, but it was not until 1997 in the USA and in 1998 in Europe, when the first anti-tumor monoclonal antibody, namely rituximab against B-cell type non-Hodgkin lymphomas, was approved for clinical treatment. The mechanisms of action as well as the therapeutic use of rituximab are described in detail in forthcoming chapters. After rituximab several antibodies have received approval for clinical use, and tens are in clinical trials. The antibodies accepted for cancer therapy are listed in Table 2.

Table 2. Clinically used monoclonal antibodies in treatment of cancer. (EGFR=Epidermal growth factor receptor, VEGF=Vascular endothelial growth factor, AML=Acute myelogenous leukemia).

Antibody	*Trade name*	*Approved*	*Specificity*	*Indication*
Rituximab	Mabthera/Rituxan	1997	CD20	B-NHL
Trastuzumab	Herceptin	1998	HER2/neu	Breast cancer
Gemtutzumab	Mylotarg	2000	CD33-calicheamicin	AML
Alemtuzumab	MabCampath	2001	CD52	CLL
Ibritumomab tiuxetan	Zevalin	2002	CD20-^{90}Yttrium	B-NHL
Tositumomab	Bexxar	2003	CD20-^{131}Iodine	B-NHL
Cetuximab	Erbitux	2004	EGFR	Colon carcinoma
Bevacizumab	Avastin	2004	VEGF	Colon carcinoma

If target antigen distribution in normal cells is limited, monoclonal antibody triggered reactions are often specific and cause only rarely systemic toxicity. If side effects are encountered, they are usually relatively mild, and are often related to the first injection only. The most typical side effects of commercially available mAbs include fever, chills, vomiting or rashes.

In addition to plain antibodies, also conjugated antibodies are produced for therapeutic use against cancer. In conjugated antibodies, the tumor specific antibody acts as a carrier of chemotherapeutic, toxic or radioactive agents (Table 2).

2.5 NON-HODGKIN LYMPHOMAS

Non-Hodgkin lymphomas are a heterogeneous group of malignancies that originate from the lymphoid system. The incidence of NHLs has increased during the last decades. NHL was the sixth most frequent type of cancer in men and the seventh in women in Finland in 2005 (Finnish cancer registry, 2005). There were about 10 new cases per 100,000 individuals each year.

Non-Hodgkin lymphomas are clonal malignant expansions of B or T cells of various stages of maturation. World Health Organization (WHO) classification divides NHLs into 30 different entities based on cellular morphology and cell lineage (Table 3) (Harris 2001). T/null-cell NHLs are rare and B-cell NHLs comprise nearly 90% of all NHLs (The Non-Hodgkin's Lymphoma Classification Project 1997). Based on different clinical behavior NHLs can be divided into indolent and aggressive types (Hiddemann et al. 1996). Indolent lymphomas have a relatively good prognosis with a median survival of 10 years, but advanced stages are considered incurable (at the time of diagnosis most patients have an advanced stage disease). The most frequent type of indolent lymphomas is follicular lymphoma. Aggressive NHLs grow rapidly and would often be fatal within two years if not appropriately treated (Chan 2001). The 5-year survival of patients with aggressive NHL is shorter than of those with indolent disease, but about half of the patients with aggressive NHL are cured. The most frequent subtype of aggressive lymphomas is diffuse large B-cell lymphoma (DLBCL).

Table 3. Basic characteristics of the most common types of non-Hodgkin lymphomas. The data is adapted from The Non-Hodgkin's Lymphoma Classification Project (The Non-Hodgkin's Lymphoma Classification Project. 1997) and bases on a large multicenter study conducted between 1988 and 1995 when rituximab and other novel treatment regimens were not available. (MALT=Mucoid associated lymphoid tissue, CLL= Chronic lymphatic leukemia, OS=Overall survival, FFS=Failure free survival).

Diagnosis	Incidence (%)	Age (year)	Female/ Male (%)	5-year OS (%)	5-year FFS (%)
Diffuse large B-cell	30.6	64	45/55	46	41
Follicular	22.1	59	58/42	72	40
Grade 1	9.5				
Grade 2	6.2				
Grade 3	6.4				
Marginal zone B-cell, (MALT)	7.6	61	55/45	74	60
Small B-lymphocytic (CLL)	6.7	65	47/53	51	25
Mantle	6.0	63	26/74	27	11
Peripheral T-cell	7.0	56	44/56	26	24
All other types	20.0				

PATHOGENESIS

The activation of oncogenes is essential for the development of lymphoid malignancies. Proto-oncogenes are normal genes usually related to proliferation, cell cycle control, or apoptosis. They have a potential to become oncogenes after amplification, point mutations, or chromosomal translocation. Oncogenes often modify a signaling pathway leading to an altered relay of extracellular messages to nucleus. Over- or underexpression, or inappropriate control of pathway components leads to dysregulation of cell growth. Distinct oncogenes have been associated with certain types of NHLs. For example, the t(14;18)(q32;q21) translocation leading to increased expressions of the anti-apoptotic protein Bcl-2 has been associated with FL, t(11;14)(q13;q32) translocation leading to cyclin D1 overexpression has been associated with mantle cell lymphoma (MCL), and

t(8;14) translocation leading to constitutive expression of *myc* gene has been associated with Burkitt lymphoma. The absence or presence of these translocations has been utilized as markers in diagnostics, and an intentional modulation of these oncogenes or pathways may offer therapeutical possibilities.

In some cases, NHLs are associated with chronic inflammatory diseases such as Sjögren's syndrome, celiac disease or rheumatoid arthritis, and sometimes with chronic infections caused by pathogens such as Epstein-Barr virus (Burkitt, AIDS-related, primary central nervous system and some other lymphomas) and Helicobacter pylori (gastric MALT lymphoma). Also immune suppression e.g. in the case of HIV infection and in high-dose posttransplant immune suppression therapy, has been shown to be associated with increased incidence of NHL.

2.6 FOLLICULAR LYMPHOMA

FL accounts for about 22 to 35% of all malignant lymphomas of adults in the Western world being the second most common type of NHL. Most patients are over 50 years of age at the time of diagnosis. No difference in the incidence between genders exists, but FL is twice as common in Caucasians as in Afro-Americans.

FL is derived from germinal center B lymphocytes. A morphological examination of excisional biopsy specimens usually provides the diagnosis. The diagnosis is based on morphology, immunophenotype, and genetic and clinical features of the disease. In the WHO classification (Harris 2001) FL is subdivided into 3 grades depending on the relative number of centroblasts in the lymphoma samples (Table 3). Grade 3 is often further subdivided into 3a and 3b, in which solid sheets of centroblasts can be observed. Excluding the more aggressive Grade 3b disease, FL is considered an indolent disease. It typically presents with slow progression and painless peripheral lymphadenopathy. Many patients are relatively asymptomatic at the time of diagnosis, even if the disease has progressed to an advanced stage. Bone marrow involvement in FL is frequent, and splenomegaly is seen in about 30 to 40 % of patients. Otherwise extranodal involvement is rare at the early stage but relatively common during the later course of disease. Systemic "B-symptoms" such as fever, night sweats, and weight loss are generally associated with a heavy tumor burden.

FL usually responds well to primary treatment, but relapses are common. Over the clinical course the disease typically develops resistance to therapy or transforms into the more aggressive DLBCL form. Excluding patients with localized disease, FL is considered to be incurable. The median survival is 8 to 10 years.

If diagnosis needs confirmation, cytogenetic studies can be performed. The t(14;18) (q32;q21) translocation has been identified in 85 to 90% of FLs (Horsman et al. 1995, Rowley 1988). It results in the overexpression of Bcl-2 and protection from apoptosis. The translocation transfers the *bcl-2* gene to the immunoglobulin heavy chain gene locus,

where it is placed under the immunoglobulin gene enhancer. The new placement of the gene leads to constitutively high expression of Bcl-2. The Bcl-2 overexpression by itself it is not sufficient to initiate tumorigenesis. An accumulation of other genomic alterations is needed for a malignant transformation.

EVALUATION OF PROGNOSIS

Staging

One of the most important factors with respect to selection of appropriate treatment and evaluation of prognosis is the stage of the disease. The staging of FL is performed according to Ann Arbor classification. The system is based on the distribution and number of involved sites and presence or absence of extranodal disease. Stages I and II represent an early stage disease on the same side of the diaphragm and stages III and IV an advanced stage disease. The staging is often accompanied by an appendix A or B indicating the absence or presence of B-symptoms including fever (over 38 °C without relation to any infection), night sweats, and loss of body weight (>10% / 6 months) (The Non-Hodgkin's Lymphoma Classification Project. 1997).

Prognostic indices

The outcome of patients has mainly been predicted using clinical prognostic indices. The International Prognostic Index (IPI) was initially designed for aggressive NHLs, but it has also been applied for FL. To calculate the IPI value age, serum LDH level, performance status, Ann Arbor stage, and the number of extranodal sites of disease are evaluated. The fact that the IPI score can classify only 10 to 15% of FL patients into a group with poor prognosis (IPI score > 3) diminishes the prediction power.

In order to recognize better those FL patients who need more intensive treatment, a new prognostic factor model Follicular Lymphoma International Prognostic Index (FLIPI) was proposed (Solal-Celigny et al. 2004). The five factors of FLIPI-index are: age (>60 years), hemoglobin level (<120 g/l), lactate dehydrogenase (LDH) level (above normal), stage of disease (I-II, vs. III-IV), and the number of nodal sites (≥ 4 vs. <4). The index divides the patients into three risk groups: low risk patients (0-1 adverse factor; 36% of patients; 5-year OS 90.6%), intermediate risk patients (2 factors; 37% of patients; hazard ratio (HR) of 2.3; 5-year OS 77.6), and poor risk patients (> 3 adverse factors; 27% of patients; HR of 4.3; 5-year OS 52.5).

TREATMENT OF FOLLICULAR LYMPHOMA

Early-stage disease

FL patients with an early-stage disease (I or II) are usually treated with radiation therapy. A typical dose is about 26 Gy, but also a low dose radiation (4 Gy or 2x 2 Gy) can produce excellent palliation (Haas et al. 2003). The toxicity of this treatment is reasonably limited and about half of the patients remain free of relapses for more that 10 years

(Wilder et al. 2001). Relapses after 10 years are rare, and most of the patients surviving the first 10 years die of other than lymphoma or treatment-related causes.

Advanced-stage disease

Because some patients with an advanced-stage disease (stage III and IV) are also asymptomatic, the first-line therapy for advanced FL varies from observation to combination chemotherapy or immunochemotherapy. For asymptomatic patients with a slowly-growing disease a watch and wait period following diagnosis may be the best option. This is a possible choice, because chemotherapy is not curable, and because the use of it affects the quality of life, contains a potential risk for myelosuppression, chronic fatigue, and secondary leukemia. In some studies no significant differences were found in the overall survival of low-grade NHL patients between an initial observation policy and an interventional approach (Ardeshna et al. 2003).

In aggressive advanced stage FL chemotherapy is extensively used. No standard chemotherapy regimen exists, but monotherapies such as chlorambucil (with or without prednisone) or cyclophosphamide, or polychemotherapies such as CVP (combination of cyclophosphamide, vincristine and prednisone), and CHOP (combination of cyclophosphamide, doxorubicin, vincristine, and prednisone) have been widely used.

Actively treated patients are followed every 6 to 12 months with clinical examination and computer tomography for 5 to 10 years (Jyrkkiö et al. 2007).

2.7 RITUXIMAB

Rituximab (Mathera®, Rituxan®) is a chimeric anti-CD20 monoclonal antibody. It consists of heavy and light chain variable regions of a murine anti-human CD20 mAb (2B8) and human IgG1/κ constant regions. The mouse part contains the antigen specificity and the human part acts as a trigger of effector systems and diminishes the risk of anti-antibody rejection. Rituximab is produced in Chinese hamster ovarian cells.

CD20

The target antigen of rituximab is CD20. It is a tetraspan cell surface molecule with an extracellular loop of 44 amino acids (Tedder, Engel 1994). The molecular weight is 33-37 kDa. The function of CD20 is not known but the non-glycosylated molecule is phosphorylated and may participate in B-cell growth via intracellular signaling or by serving as a calcium channel. MS4A1 (Membrane-spanning 4-domain, group A, member 1), the gene coding for CD20, is located on chromosome 11q12-13.

CD20 is specifically expressed in B lymphocytes (Tedder, Engel 1994). Over 90% of B cell NHLs express CD20, but the expression density is much lower on CLL cells. CD20 is absent from B-lymphoid stem cells but is highly expressed (about 100000 molecules/cell) by cells from the pre-B-stage throughout B cell maturation. CD20 expression is lost again in mature Ab-secreting plasma cells. The expression profile that

CD20 has is from the immunotherapy point of view almost ideal: because CD20 is not expressed by stem cells, a new B cell population can develop after anti-CD20 antibody treatment, and because CD20 is not expressed by plasma cells the treatment does not significantly affect immunoglobulin levels. Other benefits for immunotherapy are that CD20 does not become significantly shed from the cell surface or become internalized after ligand binding. This is a fundamental feature, because the loss of expression of the target antigens after first rounds of treatment has been one of the major problems in the development of new monoclonal antibody therapies for cancer.

CLINICAL EXPERIENCE

Single agent rituximab has been approved for the treatment of relapsed advanced stage FL. The combination therapy of rituximab with CVP chemotherapy has the indication for the treatment of advanced stage FL, and the combination therapy with CHOP for the treatment of DLBCL. In the first clinical trials rituximab monotherapy resulted in response rates of 40 to 60% for FL-patients with relapsed advanced stage disease and response rates of 50 to 70% for previously untreated patients with advanced stage disease (Colombat et al. 2001, McLaughlin et al. 1998). Despite the high initial response rates, the majority of patients still relapsed after standard rituximab therapy. Therefore, the focus turned soon towards combining rituximab with various chemotherapy regimens. Phase III trials showed that a combination of rituximab with CVP or CHOP significantly improved both the primary response rates (to 81-97%) and the outcome of previously untreated FL patients (Hiddemann et al. 2005, Marcus et al. 2005). In these trials PFS times of rituximab (R) -CVP and R-CHOP-treated patients were significantly longer than those of patients treated with the respective chemotherapy regimens (median PFS of R-CVP-treated patients was 32 months versus CVP-treated 15 months, and median PFS of R-CHOP-treated patients was 50 months versus CHOP-treated 15 months). In relapsed patients, the response rates and the PFS values were slightly lower than in previously untreated patients, but still significantly better than in chemotherapy treated patients (Forstpointner et al. 2004, van Oers et al. 2006)).

Maintenance therapy refers to therapy given after successful primary treatment to maintain the achieved remission. FL is a good candidate for maintenance therapy because of the indolent course of the disease, and because the efficacy and favorable side-effect profile of rituximab make it a very suitable agent for maintenance. In recently published phase III trials relapsed FL patients were first treated with CHOP or R-CHOP, or with FCM or R-FCM, and then randomized for maintenance rituximab therapy or observation (Forstpointner et al. 2006, van Oers et al. 2006). Maintenance therapy gave a significant survival benefit in both studies for both chemotherapy and immunochemotherapy-treated patients.

Adverse effects

The treatment with rituximab is usually well tolerated (Kimby 2005). Rituximab induces a 6-month long lymphopenia in most patients, but a full recovery is usually seen in 9 to 12

months. Adverse hematological effects or opportunist infections are rarely seen. Infusion-related symptoms like chills, fever, nausea, headache, and general weakness are common (in about 85% of patients) but usually mild and restricted to the first infusion only. They are thought to be related to the release of cytokines such as IL-6 and TNFα. More severe infusion-related reactions such as bronchospasm, angioedema, acute lung injury, and hypotension occur in approximately 10% of patients, but also these reactions are usually reversible and treatable. However, also very severe consequences of infusion-related reactions have been reported. They include pulmonary infiltrates, acute respiratory distress syndrome and cardiovascular events. In some, although very rare, cases they have been fatal.

2.8 GENE EXPRESSION PROFILING IN FL

Despite of new treatment regimens the clinical course of FL is still very variable and difficult to predict. The weakness of clinical prognostic indices is that they don't provide any information on the biology of tumors. During recent years the gene expression profiling with microarray technology has provided a method to obtain an overview on the biology of tumors and revealed factors applicable for risk stratification in FL. Husson and his colleagues compared FL cells to normal germinal center B-cells. They found differences in the expression of some transcription factors, cell cycle regulators, and genes involved in cell-cell interaction (Husson et al. 2002), but did not compare the results to the disease outcome. Bohen et al. studied rituximab monotherapy treated FL-patients (Bohen et al. 2003) and compared gene expression profiles of tumors to responder vs. non-responder distinction. They found that differentially expressed genes between the groups were often related to cellular immune responses, cytokines, complement, and T-cell receptor signaling. In a large study by Dave and colleagues tumor samples from chemotherapy treated patients revealed two signatures of coordinately regulated genes that together formed a predictor of survival (Dave et al. 2004). Emphasizing the role of microenvironment and immunological feature, many of the best differentiating genes were expressed by tumor infiltrating T cells, macrophages or dendritic cells, rather than the lymphoma cells.

In recent years many studies have investigated the contribution of specific cell subsets in the immunologic regulatory network of tumor microenvironment. A high number of tumor infiltrating T cells (Alvaro et al. 2006b) especially regulatory T cells (Carreras et al. 2006) has been shown to be associated with improved survival in response to chemotherapy. In contrast macrophages (Farinha et al. 2005), especially STAT-1 expressing macrophages (Alvaro et al. 2006a), CD57+ cells (Alvaro et al. 2006b), and T helper cells (Glas et al. 2007) in the tumor tissue have been associated with a poor prognosis. To date, however, no models are available to predict the outcome in response to immunochemotherapy.

3 AIMS OF THE STUDY

General aims of this study were to analyze how rituximab kills lymphoma cells and how factors related to mechanisms of action of rituximab or biology of tumors correlate with sensitivity of lymphomas to immunochemotherapy.

Specific aims were:

1) to evaluate the efficiencies of different effector mechanisms of rituximab (I)
2) to study the role of complement regulatory proteins as potential immunotherapy escape mechanisms of lymphomas (I & III)
3) to study complement activation in blood during and after rituximab infusion (II)
4) to study the effect of intravenously administered rituximab on CNS lymphoma (II)
5) to study if the expression of CD20 and complement regulators in lymphoma cells correlate to treatment outcome of FL patients (III)
6) to study whether differences in gene expression profiles of tumor samples correlate to treatment outcome of FL patients, and if the differentiating genes reveal potential tumor surveillance strategies (IV)

4 MATERIALS AND METHODS

4.1 GENERAL

CELL LINES (I)

Human lymphoma cell lines were grown in RPMI 1640 medium (Gibco Laboratories, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Gibco), penicillin (10 U/ml), streptomycin (100 μg/ml), and 2 nM l-glutamine (Nord Cell, Bromma, Sweden). Cells were grown to the late logarithmic phase before used for cell killing assays.

The cell lines used were HF-1.3.4 and Raji. HF-1.3.4 originally derives from a patient with FL and was obtained from Dr. Matti Kaartinen (Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Finland). Raji is a commercially available Burkitt lymphoma cell line, which we received from Professor Leif Andersson (Department of Pathology, Haartman Institute, Helsinki, Finland).

ANTIBODIES AND OTHER REAGENTS

Antibodies used in this study are shown in Table 4.

OTHER CHEMICALS (I)

N-formylmethionyl-leucylphenylalanine (N-FMLP) and phorbol myristate acetate (PMA), both from Sigma Chemicals (St. Louis, MO), were used to stimulate effector cells in cell killing assays.

COMPLEMENT COMPONENTS AND SERA (I)

Normal human serum was used as a source of C. It was prepared from coagulated blood of healthy volunteers and stored in aliquots at -70°C until used. To study CDCC, C9-deficient serum (C9DS) was used instead of NHS. C9DS was prepared by absorbing NHS with Sepharose-coupled-anti-C9 antibody. When C system was desired not to be active, heat-inactivated human serum (HIS) was used. It was prepared by incubating NHS for 30 min at 56°C.

4.2 CYTOTOXICITY ASSAYS

COMPLEMENT CYTOTOXICITY ASSAY (I)

To study direct complement-mediated cytolysis ^{51}chromium-labeled lymphoma cells were incubated for 30 minutes with rituximab and varying concentrations of NHS. The optimal rituximab concentration for cytolysis assays was found to be 10 μg/ml. Portions of supernatants were removed, and released radioactivity was counted in a gammacounter. All experiments were performed for at least 4 times. Background was obtained by measuring the release of ^{51}Cr from samples incubated only with NHS and

Table 4 Antibodies used in this work.

Antigen	*Antibody*	*Source*	*Study*
Human	**Human-mouse chimeric Ab**		
CD20	Rituximab (IDEC-C2B8)	Gift from IDEC Pharmaceuticals, Dr. Ilkka Ervaskivi, Roche, Finland	I
Human	**Mouse mAb**		
CD19	Anti-CD19	Dakopatts, Glostrup, Denmark	II
CD20	Anti-CD20cy	Dakopatts, Glostrup, Denmark	II
CD20	AP-conjugated anti-CD20	BD Biosciences	III
CD20	PerCP-conjugated anti-CD20	BD Biosciences	III
CD31	Anti-CD31	Novocastra Laboratories Ltd., UK	IV
CD46	GB24	Gift from Prof. John P. Atkinson (Washington University School of Medicine, St. Louis, MO)	I
CD46	10L46	Immunotech S.A, Marseille, France	I
CD46	FITC-conjugated anti-CD46	BD Biosciences	III
CD55	Bric216	Bioproducts Laboratories, Elstree, UK	I
CD55	APC-conjugated anti-CD55	BD Biosciences	III
CD59	Bric229	Bioproducts Laboratories, Elstree, UK	I
CD59	PE- conjugated anti-CD59	BD Biosciences	III
C5b-9 neoantigen	Wu 7.2	Gift from Dr. Reinhard Würzner, University of Innsbruck, Austria	I
Epha1	Anti-Epha1	Santa Cruz	IV
Smad1	Anti-Smad1	Santa Cruz	IV
Human	**Rabbit polyclonal ABs**		
C1q	Anti-C1q (IgG)	Dakopatts, Glostrup, Denmark	I
C3c	Anti-C3 (IgG)	Dakopatts, Glostrup, Denmark	I
Secondary Antibodies			
FITC-conjugated goat anti-rabbit Ig		Jackson ImmunoResearch Lab. Inc., West Grove, PA	I
FITC-conjugated rabbit anti-mouse Ig		Jackson ImmunoResearch Lab. Inc., West Grove, PA	I

test buffer. To achieve the total lysis reference, the cells were incubated with 0.1 % Nonidet P-40 detergent.

To evaluate the effect of C regulatory molecules, neutralizing antibodies against CD46 (25 μg/ml of GB24), CD55 (12.5 μg/ml or 25 μg/ml of Bric216), or CD59 (20 μg/ml of Bric229) were added to the solution along with rituximab. A detailed description of the C cytolysis assays is in the paper I.

ANTIBODY AND COMPLEMENT-DEPENDENT CELLULAR CYTOTOXICITY ASSAYS (I)

To study rituximab-induced ADCC ^{51}Cr-labeled lymphoma cells were incubated for 4 hours with rituximab, PBL and HIS. The contribution of CDCC was studied by replacing HIS with C9DS. The increase in cytolysis after replacement of HIS with C9DS was taken to represent CDCC. FMLP or PMA was used for ADCC and CDCC assays to stimulate PBLs. Analogously to C cytolysis assays portions of supernatants were removed and counted in a gammacounter. The background values were obtained by measuring the release of ^{51}Cr from similar samples in the absence of rituximab. To achieve the total lysis reference, the cells were incubated with 0.1 % Nonidet P-40 detergent.

4.3 IMMUNOLOGICAL TECHNIQUES

IMMUNOFLUORESCENCE MICROSCOPY (I)

To demonstrate and visualize rituximab-mediated C activation, 10^5 HF-1.3.4 and Raji lymphoma cells were incubated with various antibodies (i.e. rituximab and in some experiments with antibodies against C regulators) and NHS, centrifuged onto cytospin slides, fixed with 4% paraformaldehyde, washed, and treated with BSA to prevent nonspecific binding of antibodies. The cells were incubated with antibodies against C components, whose deposition was finally analyzed with an immunofluorescence microscope. Detailed description of the assay is provided in paper I.

DETECTION OF RITUXIMAB-INDUCED APOPTOSIS (I)

TUNEL (Tdt-mediated dUTP Nick-End Labeling) apoptosis detection system (Promega, Madison, WI) was used to analyze apoptosis after exposure of lymphoma cells to rituximab. The fluorescein-12-dUTP-labeled DNA fragments, representing apoptosis, were visualized by immunofluorescence microscopy after a 24-hour incubation period. The proportion of apoptotic cells was determined by counting the positively stained and unstained cells. Detailed description of the method is provided in paper I.

FLOW CYTOMETRY (I & III)

Flow cytometry was used for analysis of C regulator expression on lymphoma cell lines and for analysis of CD20 and C regulator expression on tumor samples of FL patients.

In paper I HF-1.3.4 and Raji cells were first pretreated with HIS to prevent non-specific immunoglobulin binding and then with mAbs directed against CD46 (10L46), CD55 (Bric216), or CD59 (Bric229). After treatment with FITC-conjugated secondary

antibodies, the intensity of immunofluorescence was analyzed and quantified using a Coulter Profile II (Coulter Electronics, Luton, UK) flow cytometer with a standard filter set-up. Detailed description of the method is provided in paper I.

The immunofluorescence stainings of patient samples were performed in the context of routine lymphoma diagnostics. Fresh lymphoma cells were separated from tumor tissue with fine needle, washed with culture medium (RPMI 1640), and centrifuged to remove dead cells. One million viable cells were incubated for 30 minutes with conjugated mAbs directed against CD20, CD46, CD55, or CD59. Immunofluorescence was analyzed and quantified using the FACSCalibur Becton Dickinson (Franklin lakes, NJ, USA) flow cytometer and WinMDI 2.8 program. Detailed description in paper III.

IMMUNOHISTOCHEMISTRY (IV)

Gene expression profiles of lymphoma tissues were analyzed with an mRNA microarray (as described in section 4.4). The results were verified and the localization of interesting proteins was evaluated by immunohistochemical stainings. Formalin-fixed, paraffin-embedded sections from tumor samples were pretreated in an autoclave with 0.01 mol/l sodium citrate, washed, and stained with anti-EphA1, anti-Smad1 and anti-CD31 antibodies. Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA) were used to complete the stainings. The immunoreactions were visualized with 3-amino-9-ethylcarbazole, and the slides were counterstained with hematoxylin. Detailed description of the method is provided in paper IV.

EVALUATION OF C COMPONENT LEVELS AND EXPRESSION OF CD19 AND CD20 ON LYMPHOCYTES (II)

To determine the concentration of C activation product C3a-desArg in plasma and in CSF the Quidel C3a Enzyme Immunoassay -kit (Quidel Corp., La Jolla, CA) was employed. Absorbances of samples were analyzed using a Titertek Multiscan spectrophotometer (Labsystems, Espoo, Finland). The cerebrospinal fluid albumin levels were determined using LC-Partigen® Albumin plates (Behring Diagnostics, Marburg, Germany), and serum C3 levels using the Behring Nephelometer Analyzer (Marburg, Germany). The expression of CD19- and CD20-antigens on blood cells and CSF lymphocytes were analyzed by immunoperoxidase staining. Detailed methods are described in paper II.

RITUXIMAB CONCENTRATION IN PLASMA AND CEREBROSPINAL FLUID (II)

To determine the concentrations of rituximab in plasma and CSF, a validated immunoassay was employed. A 96-well microtiter plate was coated with a polyclonal goat anti-rituximab antibody. Serial dilutions of patient serum or CSF were added to the wells and a goat anti-human IgG conjugated with horseradish peroxidase was used for detection. Known amounts of rituximab were used to make a standard curve on each plate. Color was developed by adding substrate to the wells and the absorbances directly proportional to the concentration of rituximab were read by photometric colorimetry.

4.4 MICROARRAY TECHNIQUES

RNA ISOLATION, PURIFICATION AND ANALYSIS OF INTEGRITY (III & IV)

Total RNA was isolated and purified from freshly frozen tumor samples employing a combination of Trizol (Life Technologies, Rockville, MD, USA) and RNeasy column purification (Qiagen) technologies. Detailed protocol can be found from the Supplementary data section of paper IV. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). Reference RNA was derived from a panel of eleven tumor cell lines.

GENE EXPRESSION PROFILING AND DATA ANALYSES (III & IV)

Isolated RNAs (20μg) were converted to fluorescently (cyanine 3-cDNA or cyanine 5-cDNA) labeled cDNA using Agilent Fluorescent Direct Label kit. The resulting reference and test cDNA samples were combined, purified, and hybridized to the Agilent Human 1A oligo microarray containing 18716 features. The microarrays were scanned with a dual laser-based Agilent microarray scanner.

Before hierarchical clustering and supervised analysis the data were pre-processed using Feature Extraction software (version A.6.0, Agilent Technologies). Detailed descriptions on data pre-processing and analysis are given in Supplementary Data section of paper IV. In supervised analysis the samples were first divided into different groups based on clinical information. Weighted voting algorithm and two-tailed t-test were used to identify differentially expressed genes, and only the genes showing significant differences in expression by both methods were further analyzed. For special analysis mRNA expression levels of CD20, CD46, CD55, and CD59 were calculated from microarray data of 23 primary R-CHOP treated patients. Detailed description of the method is provided in papers III and IV.

QUANTITATIVE REAL-TIME PCR (IV)

To verify the microarray results expressions of appropriate mRNAs were measured by real-time RT-PCR with the TaqMan methodology using a Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The detailed description of the method is available in the Supplementary Data section of paper IV.

STATISTICAL ANALYSES (III & IV)

Chi-square test and Mann-Whitney test were used to compare the baseline characteristics and expression of mRNA or protein levels. To evaluate the correlation between the data from microarray, quantitative PCR, and flow cytometry, Spearman correlation coefficient (r_s) was calculated. Survival rates were estimated by the Kaplan-Meyer method and the differences were compared by the log rank test. Cox univariate test was used to assess the prognostic value of studied parameters on time to treatment failure (TTF) or PFS.

TTF was determined as a period between the first day of therapy and the date of the documented progression or lack of response, first relapse, or death for any cause. PFS was determined as an interval between the first day of therapy and the date of relapse. These analyses were carried out using the SPSS software for Macintosh or Windows (SPSS, Inc., Chigaco, IL). Detailed descriptions are provided in papers III and IV.

5 RESULTS

5.1 EFFECTOR MECHANISMS OF RITUXIMAB (I)

THE APPROACH

Rituximab was demonstrated to kill CD20-positive lymphoma cells by multiple mechanisms, including activation of the C system, recruitment of cytotoxic cells, and induction of apoptosis (Reff et al. 1994). Because not all patients respond to rituximab treatment, even if the lymphomas express CD20 molecules, a more complete understanding of the effector mechanisms was needed.

THE ABILATY OF RITUXIMAB TO INDUCE CDC, ADCC, CDCC, AND APOPTOSIS *IN VITRO*

To analyze the sensitivity of HF-1.3.4 and Raji lymphoma cell lines to rituximab-mediated C killing the cells were treated with rituximab and NHS. The studies with optimized rituximab concentration (10 μg/ml) demonstrated C-mediated killing of 48% of HF-1.3.4 cells, and 19% of Raji cells.

To study the ability of rituximab to induce ADCC, Raji and HF-1.3.4 cells were incubated with different concentrations of rituximab, freshly isolated peripheral blood leukocytes (Effector vs. target ratio 10:1) and HIS. Following a four-hour incubation only a 10% cytolysis of HF-cells and 6% lysis of Raji cells were observed, and, the absence of rituximab did not affect the killing. N-FMLP or PMA were used to stimulate effector cells, but only a minor increase (19%) in the lysis of HF-cells was observed after PMA stimulation. No effect was seen in Raji cells or HF-cells after FMLP stimulation.

To examine the role of CDCC, the same test protocols as in the ADCC assays were used except that HIS was replaced with C9DS. No significant killing was observed when PBLs were unstimulated or when stimulated with PMA (lysis of HF-1.3.4 cells was about 12% and that of Raji cells about 15% under both conditions). FMLP stimulation enhanced the lysis of HF-1.3.4 cells to 24%.

Induction of apoptosis by rituximab was studied using the TUNEL apoptosis detection system. Following an overnight incubation with rituximab, apoptosis related DNA fragmentation was observed in 40% of HF-1.3.4 cells, but in only 8% of Raji cells.

Taken together, these results suggest that the cytotoxic effect of rituximab *in vitro* is mediated mainly through direct CDC. Neither ADCC nor CDCC were significantly enhanced by rituximab, but rituximab did induce apoptosis of HF-1.3.4 cells.

RITUXIMAB-INDUCED C COMPONENT DEPOSITION ON LYMPHOMA CELLS

Deposition of C components on lymphoma cell membranes was studied by treating HF-1.3.4 cells with rituximab and NHS. The samples were stained with antibodies against C1q, C3c and C5b-9. Positive C1q cell membrane staining showed that rituximab can trigger the classical complement pathway, and the deposition of C3 on cell membranes confirmed an effective rituximab-induced C activation. After staining with anti-C5b-9 antibody (Wu 7.2) MAC complexes were regularly visualized on cell membranes. As an indicator that the formation of MAC complexes had led to C-mediated cell killing, normal cell morphology was usually lost.

5.2 NEUTRALIZATION OF C REGULATORS IN VITRO (I)

Surface expression of CD46, CD55, and CD59 on HF-1.3.4 and Raji lymphoma cells was quantified by flow cytometry analysis after staining with respective antibodies. Both cell lines expressed strongly CD46, CD55, and CD59 on their surfaces. The HF-1.3.4 cells displayed greater mean fluorescence intensities for all complement regulators compared to Raji cells. In both cell lines CD59 exhibited the strongest expression and CD55 the weakest.

To examine the contribution of the C regulators for protection from C lysis the HF-1.3.4 and Raji cells were exposed to rituximab, NHS, and neutralizing antibodies against CD46, CD55 or CD59. After treatment with antibodies against CD59 (Bric229), DAF (Bric216) or MCP (GB24), loss of continuity of cell membranes and increased cell damage were observed. Neutralization of CD46 by the GB24 antibody increased rituximab-mediated CDC from 48% to 82% in HF-1.3.4 cells and from 19% to 40% in Raji cells. Neutralization of CD55 with Bric216 resulted in 81% lysis of HF-1.3.4 and of 63% lysis of Raji cells, and neutralization of CD59 with Bric229 in 79% lysis of HF-1.3.4 and to 81% lysis in Raji cells. Neutralization of all C regulators simultaneously had no further effect to HF-1.3.4 cells (lysis level stayed at around 80%), but increased the lysis of Raji cells to 93%. C regulators thus considerably affect rituximab-mediated CDC *in vitro*. Compared to HF-1.3.4 cells Raji cells were initially less sensitive to rituximab treatment, but neutralization of CD59 equalized the difference.

5.3 COMPLEMENT ACTIVATION IN BLOOD AND CSF AFTER RITUXIMAB INFUSION (II)

THE APPROACH

In vitro studies suggested that CDC plays a key role in rituximab-induced killing of lymphoma cells. No information on the effect of rituximab on central nervous system lymphoma was available yet many patients suffer form this complication. We studied C activation in blood and in CSF at certain time points before, during, and after intravenously administered rituximab in one patient with anaplastic large cell lymphoma affecting central nervous system. Blood samples were taken before rituximab treatment

and 2, 4, 12, 24, and 48 hours after the first and second infusion and CNS samples before and 4 and 24 hours after the infusions. Both samples were also taken once after third and fourth infusion.

DISAPPEARANCE OF CD20 POSITIVE CELLS FROM BLOOD DURING RITUXIMAB INFUSION

Surface marker analysis of blood cells demonstrated that before the first rituximab infusion 41 % of blood lymphocytes were CD19-positive and 35 % were CD20-positive. Two hours after beginning of the first rituximab infusion, no lymphocytes were seen using anti-CD20 antibody. Thereafter the absolute and relative (between 0 and 3 %) levels of CD20-positive cells remained intact during the whole treatment period. Because binding of rituximab to CD20 might affect the detection of lymphoma cells with another CD20 antibody, we also stained them with anti-CD19 antibody. After the first rituximab infusions the amount of CD19 positive cells stayed below 7% in all measurements. The results suggest that both normal and malignant B-cells were rapidly and efficiently eliminated from the circulation of the patient.

C ACTIVATION IN PLASMA AFTER RITUXIMAB INFUSION

To analyze rituximab-induced C activation we measured levels of C3a-desArg, which is an activation fragment of the central C component C3. The initial C3a-desArg concentration in plasma before the first rituximab infusion was 55 ng/ml, and it increased to 138 ng/ml during the first two hours after beginning of the first rituximab infusion. Thereafter the C3a-desArg concentration decreased in two hours to the basic level and remained low in all subsequent measurements. No similar C activation was observed related to the second, third or fourth infusion.

RITUXIMAB CONCENTRATION AND C ACTIVATION IN CSF

Rituximab was also detected in CSF but the concentrations were 500 to 1000 times lower than in plasma (<0.5 μg/ml compared to max. 388 μg/ml in plasma). Before rituximab treatment 10 % of lymphocytes in CSF were CD20-positive, but rituximab had no effect on the amounts of CD20 positive cells in the CSF. Also, the radiological findings remained unchanged. Neither an immediate increase in the C3a-desArg level was seen. However, a minor and delayed C activation response was seen 24 to 48 hours (max 13ng/ml) after beginning of rituximab infusion, but it had no effect on the number of CD20 positive cells in the CSF.

5.4 EFFECT OF CD20 AND C REGULATOR LEVELS ON OUTCOME (III)

THE APPROACH

Our previous results and several other studies demonstrated that neutralization of C regulatory proteins strengthened the cell killing efficacy of rituximab. In contrast, expression of C regulators was not shown to correlate to the primary response of rituximab treatment *in vivo* (Bannerji et al. 2003, Weng, Levy 2001). To analyze whether the expression of C regulators is associated with outcome parameters, we analyzed

mRNA levels of CD20, CD46, CD55, and CD59 from pretreatment lymphoma samples of 23 R-CHOP treated FL patients and determined their association with PFS. To validate the gene expression data, we set up a prospective pilot study of 12 patients, where membrane expression levels of CD20 and C regulators were determined by flow cytometry from pretreatment lymph node biopsies used for diagnostics.

EVALUATION OF CD20, CD55, CD46, AND CD59 MRNA LEVELS FROM MICROARRAY DATA

23 R-CHOP treated FL patients were included in this study. 21 of 23 (91%) patients responded for treatment and 16 (70%) achieved complete response. Median follow-up was 55 months. Patients were divided into "long-term responders" (PFS>35 months; n=13) and "short-term responders" (PFS < 23 months; n=10) based on significantly different outcome rates. High expression levels of CD55 and CD59 in the tumor cells were more often observed among the short-term than the long-term responders (p=0.026 and p=0.077), whereas no such association was observed for the CD20 or CD46 mRNA levels. High CD55 expression levels were also shown to associate with the high FLIPI score (p=0.02) and low complete response (CR; p=0.04) rates, but not with the overall response (OR) rates.

In a survival analysis the median PFS time for patients with low CD55 was significantly longer than for those with high CD55 expression (not reached vs. 20 months, p=0.003). Also patients with low CD59 levels tended to have longer PFS than patients with intermediate or high CD59 expression (median PFS not reached vs. 21 months, p=0.09).

EVALUATION OF CD20, CD55, CD46, AND CD59 PROTEIN EXPRESSION BY FLOW CYTOMETRY

In order to verify the microarray results on C regulation and CD20 expression a prospective pilot study of 12 immunochemotherapy treated patients was performed. In this cohort 10 out of 12 patients responded to immunochemotherapy, and two patients showed a stable disease (SD). In addition, two patients received an early relapse after 6 and 13 months. Median follow-up was 23 months. On this basis we divided the patients into "failured patients" (n=4; two early relapsed patients and two with SD response) and "patients in remission" (n=8). Expression of CD20, CD46, CD55, and CD59 in cells derived from the lymphoma tissues were evaluated by flow cytometry, but no significant differences in protein levels between the two groups were observed.

To direct the analysis more specifically to FL cells, the CD20-positive population from each sample was selected and C regulator levels were compared to CD20 levels in these cells. The CD20/CD55 ratios tended to be higher among the patients in remission than in failured patients (mean 2.56 versus 1.01). The difference between the two groups became more obvious when the CD20 levels were compared to combined average C regulator levels. Also in Kaplan-Meyer estimates a trend towards a better outcome was observed in patients with a high lymphoma cell CD20/CD55 ratio (p=0.11) or CD20 vs. average C regulator level (p=0.018).

Taken together, differences in the expression of C regulators, especially of CD55, were observed between FL patients, and in various comparisons these differences were associated with outcome in response to immunochemotherapy. However, because of the relatively low number of patients in the study groups the differences did not reach statistical significance.

For one patient we could also analyze the tumor sample from the relapse. Interestingly, the expression levels of all three C regulators were higher in this relapse sample as compared to the pre-treatment sample.

5.5 ASSOCIATION OF FL GENE EXPRESSION WITH TREATMENT OUTCOME (IV)

THE APPROACH

Because the outcome to immunochemotherapy differs remarkably between individual patients, and the differences cannot be fully predicted with clinical prognostic indices, we searched for novel molecular factors correlating with outcome in response to rituximab and CHOP chemotherapy. Enough mRNA for screening gene expression profiles was available from 24 samples. Based on clearly different clinical courses the patients were divided into long-term responders (n=11, PFS; 35 to 66 months), and short-term responders (n=13, PFS < 23 months). The results were confirmed by immunohistochemistry using a validation group of 40 FL patients.

IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED BETWEEN LONG AND SHORT TERM RESPONDERS

Expression patterns of lymphoma tissues from different patients were relatively homogeneous, and the clusters arising from unsupervised hierarchical clustering did not associate with clinical parameters. We subsequently conducted a supervised learning classification and sorted the genes by their degree of correlation with the favorable versus unfavorable distinction (responder versus non-responder or long-term versus short-term responder). Weighted voting algorithm and t-test identified numerous genes whose expression varied between the groups. From all distinctive genes we randomly chose few biologically interesting genes for qPCR validation (Table 5). *smad1* (r_s=0.665, p=0.003; overexpressed in short-term responders), *epha1* (r_s=0.688, p=0.002; downregulated in short-term responders), *marco* (r_s=0.529, p=0.020; overexpressed in non-responders), and *mxi1* (r_s=0.476, p=0.038) showed correlation between PCR and microarray results, but differential expression of *cul4b* mRNA could not be verified by qPCR. The best correlating genes *smad1*, *epha1* and *marco* were chosen for further analysis.

qPCR results were compared with the outcome of patients, and considerably longer median TTF values were associated with low *smad1* (p=0.019), low *marco* (p=0.018), and high *epha1* (p=0.077) qPCR levels. The results suggest that expression of these genes in

lymphoma tissue correlates with the outcome in response to rituximab and CHOP chemotherapy.

Table 5. qPCR-validated genes associated with outcome of R-CHOP treated FL patients.

Gene	*Spearman correlation co-efficient*	*p-value*	*Effect on outcome*	*Function*
smad1	0.665	0.003	Unfavorable	Signal transduction
epha1	0.688	0.002	Favorable	Leukocyte migration
marco	0.529	0.020	Unfavorable	Macrophage scavenger receptor
mxi1	0.476	0.038	Favorable	Signal transduction

EPHA1 AND SMAD DETECTION BY IMMUNOHISTOCHEMISTRY

In order to extend the gene expression-based analyses to protein and cellular localization level, and to confirm the results, we performed immunohistochemical stainings of tumor samples for EphA1 and Smad1 proteins. The gene expression results concerning *marco* could not be verified, because no antibody for paraffin-embedded tissue was available.

Some positive Smad1 staining was observed in malignant lymphocytes, but the most prominent expression appeared to localize to vascular structures. To get more information about the vasculature of tumor tissue, and to count the vessel density in tumors, CD31 staining was performed, but no difference was found between long and short-term responders (mean 13.9 vs. 13.2 per TMA core, p=0.74).

The immunohistochemical analysis showed that EphA1 was not observed in malignant lymphocytes but in the surrounding microenvironment, including vascular endothelia and granulocytes close to high endothelial venules. The differences at the protein level between the long-term and the short-term responders were less apparent than the differences at the mRNA level. The semiquantitative grading of protein expression failed to correlate reliably with the mRNA data, but in a "hot spot" approach, a good correlation between mRNA and protein expression of Epha1 (r_s=0.628, p=0.003) was observed. Also EphA1 positive granulocyte counts were higher among long than short-term responders (mean 14 vs. 8 per TMA core, p=0.039). A trend towards a better outcome was seen among the patients having high EphA1 protein expression in their lymphoma tissue (58 vs. 21 months, p=0.259).

Because Epha1 expression was associated with the outcome at both mRNA and protein levels, we confirmed the results immunohistochemically in a validation group of 40 R-CHOP-treated patients. Although no cut-off level for a significant difference between EphA1 low and high groups could be determined, we observed a trend towards a better outcome among patients with high EphA1 protein expression (two upper tertiles versus the lowest tertile) in their lymphoma tissue (p=0.07). Of the 26 patients within the two upper tertiles, 19 (83%) were in remission (median PFS not reached), and out of 17

patients within the lowest tertile seven (41%) were in remission (median PFS 43 months). The results suggest that high EphA1 expression is associated with better PFS of immunochemotherapy treated FL patients.

6 DISCUSSION

The addition of rituximab to chemotherapies has significantly improved the treatment outcome of follicular lymphoma. The main focus of this work was to study how rituximab actually works, and which factors determine its efficacy. In addition to scientific interest, the results may provide new approaches for the prediction of the outcome and for the development of novel strategies to improve the treatment results. The work was started by a study on the effector mechanisms of rituximab *in vitro* and events after rituximab infusion in blood and CSF. During the last couple of years the first prospective trials of efficacy of immunochemotherapy were completed. Mostly due to excellent results of this therapy also the focus of the study turned towards factors related to predicting the outcome in response to immunochemotherapy.

6.1 HOW DOES RITUXIMAB WORK?

Rituximab is known to specifically recognize CD20 positive cells, but how does it exert its effect on B cell and lymphoma cells? The following chapters will discuss the events from the distribution of rituximab to CD20 binding and to the effector mechanisms recruited by rituximab. The initial prerequisite for a cytotoxic effect against a tumor is that sufficient quantities of rituximab reach the lymphoma tissue.

SERUM LEVELS AND DISTRIBUTION OF RITUXIMAB

Rituximab is usually given at dose of 375 mg/m^2. The reported half-life after first infusion in monotherapy is 76 hours and maximal plasma concentration 205.6 ± 59.9 μg/ml (Berinstein et al. 1998). In our case study the maximum rituximab concentration in blood plasma related to the first infusion was 331 μg/ml measured two days after the beginning of infusion. Achieving a relatively high concentration, this patient also responded well to the treatment. It has been speculated, whether rituximab levels would affect the outcome. In one study the levels were indeed higher in responders than in non-responders (Berinstein et al. 1998). However, the actual level depends on the amount of accessible CD20, because rituximab is consumed, when bound to circulating or cell surface CD20 molecules. Therefore we cannot know whether higher rituximab levels measured among responders were due to a lower tumor burden and fewer CD20 molecules leaving more non-consumed rituximab for measurement, or whether the metabolism of responders was more favorable leading to higher rituximab concentrations and better outcome.

Lymphomas often affect the CNS, but only very little information is available on the access of rituximab to the CSF. We also know that although rituximab has improved the overall survival of NHL patients it has not influenced the risk of CNS occurrences (Feugier et al. 2004). The results in this work showed several hundred-fold lower levels of rituximab in the CSF than in plasma suggesting that the blood-brain barrier can

effectively prevent the passage of antibodies. In line with previous clinical experience, the low levels of rituximab in the CSF caused neither a decrease in number of CD20 positive cells nor any therapeutic effect.

CD20 EXPRESSION

The importance of CD20 expression levels on tumor cells to the outcome of rituximab treatment has been investigated in various studies, but no clear association has been observed. In general, low CD20 levels in CLL and high levels in FL cells could explain their different responses to rituximab treatment but, on the other hand, more variably responding MCL and DLBCL often express even more CD20 than FLs. *In vivo* studies focusing on this question are rare, but a response to rituximab did not correlate to CD20 levels in a study on nine CLL patients (Perz et al. 2002). Instead, numerous *in vitro* studies have investigated the influence of variation in CD20 levels on CDC. A relationship between high CD20 expression by tumor cells and efficiency of CDC has been reported in some studies (Bellosillo et al. 2001, Golay et al. 2001, van Meerten et al. 2006), but not in others (Golay et al. 2000, Manches et al. 2003, Terui et al. 2006). Van Meerten and colleagues (van Meerten et al. 2006) studied cell lines transfected with different amounts of CD20 and found that CDC but not ADCC was dependent on CD20 levels. Our data showed a weak correlation between the outcome and membrane expression of CD20. The expression of CD20 was slightly higher in patients with a better outcome, but the differences were small and without a statistically significant survival benefit. If CD20 levels were of major importance, the levels of CD20 would probably be reduced in relapses. Clinical studies have shown that CD20 negative relapses are very rare in indolent lymphoma (Davis et al. 2000), but may occur in aggressive lymphoma (Davis, Czerwinski & Levy 1999, Foran et al. 2001, Kennedy et al. 2002).

6.2 EFFECTOR MECHANISMS OF RITUXIMAB

Effector mechanisms of rituximab have been under intensive research since the first promising results of this anti-cancer antibody. Better understanding of effector mechanism would be beneficial for several reasons. Effector mechanisms may e.g. reveal potential tumor escape mechanism and enable their neutralization, help to develop rituximab molecule, and give reasons to reconsider the treatment schemes: e.g. simultaneous administration of chemotherapeutics may be disadvantageous for cellular effector mechanisms.

The recruitment of cellular (ADCC), and humoral (CDC) effector mechanisms as well as their combined action (CDCC) and direct cytotoxic effects have all been found to take place at least under *in vitro* conditions. Also, the role of possible vaccine-type effects has been under discussion. Despite numerous studies no consensus has been reached over which of these mechanisms is the most important *in vivo* and how important their combined action is.

RITUXIMAB BINDING TO CD20 INDUCES APOPTOSIS

Antibody binding to CD20 has been reported to induce numerous biological effects. Most of them are related to the activities of tyrosine kinases and associated with cell cycle arrest or induction of apoptosis. There has been a wide variation in the amounts of apoptosis detected under different set-ups and in studies by different investigators (reviewed by Deans (Deans, Li & Polyak 2002)). Also in this work strong variation in apoptosis was observed between the studied cell lines (I). In many publications apoptotic effects have been significantly enhanced by cross-linking of antigen-bound antibodies with secondary antibodies (hyper-cross-linking) (Ghetie, Bright & Vitetta 2001, Pedersen et al. 2002, Shan, Ledbetter & Press 1998). The association of variable apoptotic effects with differences in the translocation of CD20 to lipid rafts has been a fundamental finding for studies on intracellular signaling (Deans et al. 1998, Polyak, Deans 2002). Lipid rafts are membrane-microdomains enriched in cholesterol, sphingolipids, and signal transduction molecules such as Src tyrosine kinases. They are thought to function as platforms for signaling. Some CD20 antibodies can translocate CD20 to lipid rafts much more efficiently than others. Besides, the lipid raft formation can be affected by a different developmental status or the activation stage of the B cell. In lipid rafts, CD20 activates Src-family tyrosine kinases lyn, fyn, lck and p75/85, which initiate apoptotic cascades (Figure 4). Tyrosine kinase activation leads to phosphorylation of phospholipase C, Ca^{2+} influx, caspase-3 activation, and apoptosis (Byrd et al. 2002, Deans et al. 1993, Deans et al. 1995, Hofmeister, Cooney & Coggeshall 2000, Pedersen et al. 2002, Shan, Ledbetter & Press 1998, Shan, Ledbetter & Press 2000). In addition to direct proapoptotic effects, rituximab has been shown to down-regulate survival pathways and sensitize lymphoma cells to apoptosis-inducing effects of other agents such as doxorubicin, cisplatin, dexamethasone, fludarabine and retinoids (Alas, Bonavida 2001, Alas, Emmanouilides & Bonavida 2001, Alas, Ng & Bonavida 2002, Di Gaetano et al. 2001, Ghetie, Bright & Vitetta 2001, Jazirehi et al. 2005, Jazirehi, Bonavida 2005).

CD20-mediated tyrosine kinase activation diminishes the activity of the p38MAPK signaling pathway. Via downregulation of IL-10 this inhibits constitutive STAT (signal transducer and activator of transcription) 3 activity and downregulates the anti-apoptotic protein Bcl-2 (Alas, Bonavida 2001). In non-AIDS-related lymphoma cell lines rituximab has been shown to downregulate the extracellular signal-regulated kinases 1/2 (ERK1/2) and NF-$\varkappa$B pathways. Down-modulation of these two major survival pathways leads to the down-regulation of Bcl-xL transcription. Downregulation of both Bcl-xL and Bcl-2 leads to sensitization of lymphoma cells to drug-induced apoptosis.

Even if *in vitro* studies demonstrated numerous apoptotic pathways, where rituximab may be involved, only limited information is available on their contribution in clinical settings. Rituximab has been shown to activate apoptosis-related caspases 3 and 9 (Byrd et al. 2002), and the ratio of caspase-related factors Mcl (myeloid cell leukemia sequence) -1 and Bax has been observed to correlate with the primary response in CLL (Bannerji et al. 2003). Unfortunately, no such information is available for NHL.

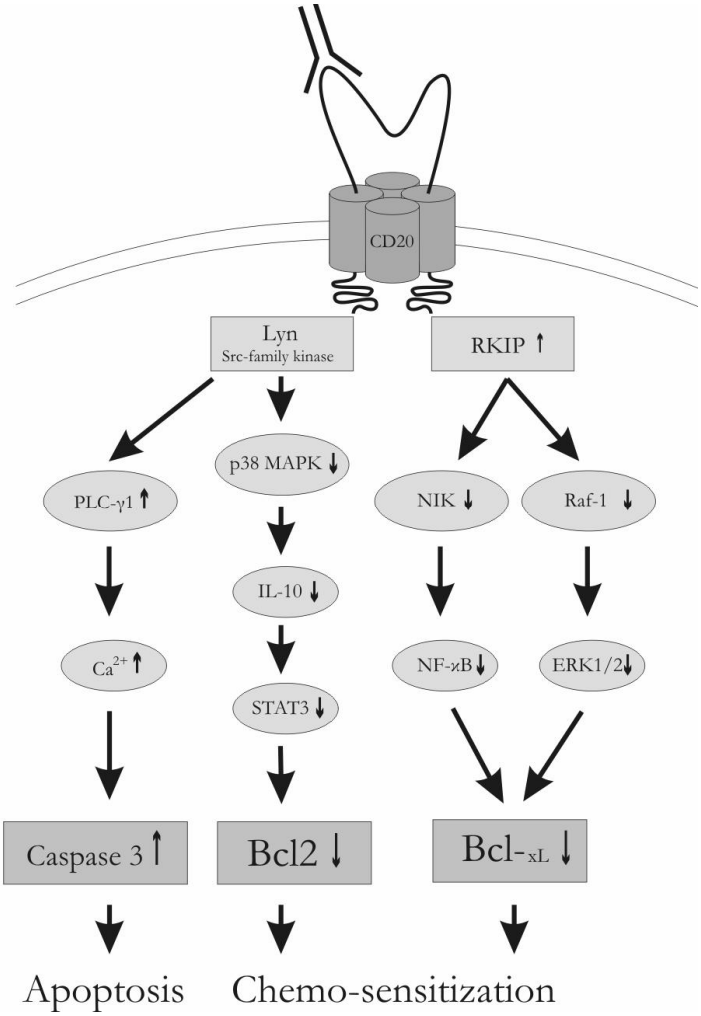

Figure 4. Rituximab-induced apoptotic and chemo-sensitizing pathways.
In lipid rafts rituximab binding can activate lyn and other Src-family tyrosine kinases to initiate apoptotic cascades via phosphorylation of phospholipase C and chemo-sensitizing cascades via diminished the activity of the p38MAPK signaling pathway. Rituximab-mediated chemo-sensitization has been shown to occur also via up-regulation of expression of Raf-kinase inhibitory protein (RKIP).

COMPLEMENT-DEPENDENT CYTOTOXICITY (CDC)

Rituximab can activate the classical complement pathway by binding the complement component C1q. The substitution of certain residues (D270, K322, P329, and P331) on the C_H2 domain of rituximab has been shown to reduce dramatically its C1q binding capacity (Idusogie et al. 2000). Other structural studies have demonstrated that binding sites for C1q and Fc-receptors are different, and that one rituximab molecule can, in principle, induce both CDC and ADCC.

This work has examined rituximab-induced complement activation both under *in vivo* and *in vitro* conditions. Different effector mechanisms of rituximab were compared *in vitro*, and CDC was demonstrated to be superior to the other effector mechanisms. We also observed deposition of C components C1q, and C3, and terminal complement

complexes on the cell surfaces of rituximab and C9-deficient serum-treated lymphoma cells. This suggests that rituximab activates the classical C pathway, and that C cascade proceeds to activation of the terminal complement pathway and the MAC formation. When normal human serum was used as a source of C, the cell morphology was lost due to direct C mediated lysis. The results are in line with several other *in vitro* studies, where effector mechanisms of rituximab were studied against B-cell lymphoma cell lines (Flieger et al. 2000, Reff et al. 1994, van Meerten et al. 2006) and fresh lymphoma cells (Bellosillo et al. 2001, Golay et al. 2000, Golay et al. 2001, Manches et al. 2003). The summary of C-related studies is provided in Table 6.

Some studies have shown that the efficiency of CDC correlates at least partly with the levels of CD20 antigen on target B cells (Bellosillo et al. 2001, Golay et al. 2001, Manches et al. 2003, van Meerten et al. 2006). In studies with CD20-transfected cell lines Van Meerten et al. demonstrated a sigmoidal correlation of CD20 expression levels to CDC. This finding suggests that effective C activation requires a threshold level of CD20 on target cells.

Activation of the C system has also been associated with the ability of anti-CD20 antibodies to translocate CD20 to lipid rafts (Cragg et al. 2003). Most strikingly B1, another anti-CD20 antibody (tositumomab), does not translocate CD20 into lipid rafts nor at all induce C-dependent lysis. Redistribution of CD20-molecules into lipid rafts leads to stronger C1q binding and activation of the C cascade (Golan, Burger & Loos 1982). The reason for this is not understood, but one can speculate that a high local density of CD20 molecules in lipid rafts is required to achieve a needed multi-point binding of C1q.

Much less information is available on the role of complement-killing *in vivo*. In an elegant animal study by Di Gaetano and her colleagues rituximab could efficiently eradicate murine lymphoma cells (EL4) transfected with human CD20 in immunocompetent mice, but rituximab was totally ineffective in C1q knock-out animals. (Di Gaetano et al. 2003). They also showed *in vitro* that rituximab does not induce significant direct apoptosis and that depletion of NK, PMN, or T-cells *in vivo* do not affect to the therapeutic effect of rituximab.

In this study C activation was evaluated before, during, and after rituximab treatment with longitudinal blood samples. The C cascade was found to be activated rapidly after beginning of the first rituximab infusion. The C3 activation product C3a-desArg concentration increased from 55 ng/ml to 138 ng/ml in two hours, but decreased soon back to the basic level. Compared e.g. to C activation levels measured in children during cardiopulmonary bypass the absolute amounts of C3a-desArg remained relatively low (Meri, Aronen & Leijala 1988), but most probably we missed the "maximum" C3a-desArg concentration because no samples were taken during the first two hours of the infusion. After subsequent rituximab infusions no such increase in C3a-desArg

concentration was observed. This is not surprising, because the surface marker analysis of blood lymphocytes showed that virtually all CD20 positive cells were already removed from circulation during the first two hours of the first infusion, and their amounts remained low in all subsequent measurements. Our patient in this study (II) did not show notable side effects, but a strong C activating potential of rituximab can also be considered as a potential cause of adverse effects. In clinical use and especially in patients with a large tumor burden rituximab can cause an extensive C activation and strong systemic effects. In a study of five patients higher C3b/c levels were found in patients with more severe side effects (van der Kolk et al. 2001). Based on this observation the authors proposed that the C system may play a key role in inducing the side effects of rituximab.

The immediate systemic reactions are mainly mediated by the anaphylatoxins C3a and C5a. They activate mast cells to release histamine, leukotrienes, prostaglandins, cytokines and other mediators. Anaphylatoxin and cytokine bursts may lead to a systemic inflammatory response. In minutes up to an hour C5a will also activate polymorphonuclear leukocytes, which may become aggregated in capillaries. The obstruction of pulmonary capillaries may lead to an ARDS (adult respiratory distress syndrome) -like pulmonary dysfunction and hypoxia.

C regulatory proteins

If CDC were an effective mechanism of the action of rituximab, neutralization of C regulatory proteins of tumor cells should enhance rituximab-induced killing. In our *in vitro* experiments neutralization of CD46, CD55 and CD59 clearly increased C lysis. Flow cytometry analysis showed that the expression of CD59 was higher than the expression of CD46 or CD55 in the studied HF-1 and Raji cell lines. In line with this the neutralization of CD59 proved to be the most effective method to enhance killing. Membrane bound C regulators have considerably influenced in rituximab-mediated killing also in other studies (Golay et al. 2000, Manches et al. 2003, Terui et al. 2006, Treon et al. 2001, Ziller et al. 2005) (Table 6). Either CD55 or CD59 has been found to be the most effective regulator, but most of the studies, especially the ones where neutralizing antibodies were used, concentrated on CD55 and CD59, and CD46 has been studied much less extensively

In our clinical study (III) we found significantly higher CD55 mRNA levels in the lymphoma tissues of patients with adverse outcome after R-CHOP therapy as compared to patients with favorable outcome. No similar differences were seen, when membrane levels of CD55 proteins were compared. However, when ratios of CD20 and CD55 were analyzed, the differences were again observed. Of the various C regulators the expression of CD55 is most actively regulated. A rapid increase in the expression levels of CD55 has been related with various biological conditions such as sprouting of neurons (Zhang et al. 1998) and activation of neutrophils (Berger, Medof 1987). Also, shedding of CD55 to the extracellular matrix *in vivo* and loss of CD55 *ex vivo* during treatment of tumor samples

Table 6. A summary of studies in which effector mechanisms of rituximab, expression of C regulators and the effect of neutralization of C regulators have been investigated. (↑=Moderate effect, ↑↑= Major effect, ↔= no significance, NS= not studied)

		Effector mechanism				*Expression of*					*Neutralization of*		
Studies with cell lines		*ADCC*	*CDC*	*CDCC*	*Apoptosis*	*CD20*	*CD46*	*CD55*	*CD59*	*CD20/ C-reg*[A]	*CD46*	*CD55*	*CD59*
Reff, 1994	Human Lymphoblastoid cell line	↑↑	↑↑	NS	NS								
Harjunpää (I)	FL and Burkitt lymphoma	↔ (PBL)	↑↑	↔	↑						↑	↑	↑↑
Golay, 2000	FL and Burkitt lymphoma (3 fresh FL samples)	↑↑ monocytes / NK	↑↑	NS	↔						↔	↑↑	↑↑
Fliegel, 2000	4 lymphoma cell lines	↑↑	↑	NS [B]	↑								
Ziller, 2005	2 NHL cell lines [C]										NS	↑↑	↑↑
Studies with fresh lymphoma samples													
Golay, 2001	33 CLL, 6 PLL, and 6 MCL					↑↑	NS	↔	↔		NS	↑↑	↑↑
Bellosillo, 2001	33 CLL, 16 MCL, 4 FL, HCL					↑↑	NS	↔	↔		NS	↑	↑↑
Manches, 2003	7 FL, 7 MCL, 7 DLBCL, and 7 SLL	↑↑ (NK) / ↔ monocytes/PBLs	↑↑	NS	↑	↔	↔	↔	↔	↑↑			
Terui, 2006	30 NHL [D]					↔					NS	↑↑	NS
In vivo studies													
Weng, 2001	29 NHL patients / (Primary response)					NS	↔	↔	↔	NS			
Bannerji, 2003	21 CLL patients / (Primary response)				↑	NS	NS	↔	↔/↑[E]	NS			
Di Gaetano, 2003	C1qa-/- and WT mice [F]	↔	↑↑	NS	↔								
Sommarhem (III) (submitted)	35 FL patients (immunochemotherapy outcome)					↔	↔	↔/↑↑[G]	↔/↑[G]	↑↑			
van Meerten, 2006	CEM cells transfected with different amounts of CD20 DNA	↑↑	↑↑			[H]							

(A) Expression of CD20 versus Average expression of CD46, CD55 and CD59.
(B) In this work CDC has obviously been indicated as CDCC
(C) Novel human αCD55 and αCD59 Abs were tested
(D) CD55-mediated resistance was overcome with siRNA
(E) CD59 levels were found to be significantly higher in tumor cells, which had survived rituximab therapy.
(F) The mice were injected with human CD20 transfected murine lymphoma cells. ADCC was studied with depletion of NK cells and neutrophils.
(G) Membrane expression / mRNA expression
(H) CD20 levels did affect the efficacy of CDC but not the efficacy of ADCC.

can also affect the measurements of protein expression. On the other hand the local amount of CD55 on lipid rafts may be more important in determining the efficiency of CDC than the total expression of CD55 on cell membranes. Furthermore, besides membrane bound CD55 also soluble CD55 has some functionally activity (Hindmarsh, Marks 1998). All these factors may explain why the mRNA expression of CD55 did not correlate that clearly to membrane expression levels in our studies.

If C regulators provided a clinically relevant escape mechanism against immunotherapy, one would expect that in possible relapses C resistant clones would be enriched and C regulator levels in relapsed tumors would be higher than in primary samples. Studies about this have not been published, but as a single finding we observed an increased expression of CD46, CD55 and CD59 in a relapsed tumor of one patient compared to his pretreatment tumor sample. Interestingly, Bannerji and his colleagues analyzed CD55 and CD59 expression levels from pre- and post-treatment samples of seven patients, whose rituximab treatment did not effectively clear CD20 positive cells from circulation (Bannerji et al. 2003). The CD55 and CD59 expressions were regularly higher in post-treatment samples suggesting that subclones expressing higher amounts of CD55 and CD59 may have been selected during rituximab treatment. It has been demonstrated *in vitro* that the treatment of lymphoma cells with sublytic amounts of rituximab can increase CD55 and CD59 expression levels, and make these cells more resistant to rituximab-mediated CDC (Takei et al. 2006). If studies in future will confirm these observations, the role of C will be strongly emphasized as a central effector mechanism of rituximab. The finding also suggests that C regulatory molecules may be determining factors for the success of immunochemotherapy.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

The Fc-part of rituximab is recognized by Fcγ-receptors of effector cells. Different Fcγ-receptors are expressed by monocytes, macrophages, NK-cells, neutrophils, and dendritic cells, but the precise contribution of various effector cells in ADCC *in vivo* has not been fully determined. Some *in vitro* studies have shown that rituximab induces ADCC in lymphoma cell lines, but in some others including our *in vitro* study (I) and animal study by Di Gaetano (Di Gaetano et al. 2003) effective antibody-dependent responses could not be induced. The differences in these results may be due to different effector cells. Especially the studies, which have focused on FcγRIIIA, have emphasized the role of NK-cells as important effectors. E.g. Manches and Golay could induce ADCC *in vitro* using monocytes and NK-cells as effectors (Golay et al. 2000, Manches et al. 2003), but neither Manches nor we could demonstrate significant rituximab-induced ADCC, when human peripheral blood leukocytes were used as effectors. On the other hand, in the absence of neutrophils, rituximab was less effective in controlling lymphoma cell growth in SCID mice (Hernandez-Ilizaliturri et al. 2003).

The importance of ADCC was clearly demonstrated *in vivo* in a xenograft model of lymphoma using Fcγ-receptor deficient mice (Clynes et al. 2000). Rituximab-mediated

tumor cell killing was significantly increased in mice lacking the inhibitory FcγRIIB, whereas the killing capability was totally lost in common γ-chain knock-out animals. It has also been observed that genetic polymorphisms of human FcγRIIIa can influence the therapeutic efficacy of rituximab. Either phenylalanine (F) or valine (V) can be expressed at position 158 of Fcγ receptor IIIa. FcγRIIIa with homozygous VV binds rituximab with a higher affinity than a receptor with phenotypes VF or FF (Cartron et al. 2002). Patients with the VV phenotype have also shown better primary responses and longer PFS times than those carrying the VF or FF phenotype (Cartron et al. 2002). In contrast to FL FcγR-polymorphism did not show predictive power in CLL (Farag et al. 2004). It also remains to be shown whether FcγRIIIa polymorphism has impact on immunochemotherapy treated FL patients. Most studies about rituximab mediated ADCC have focused on Fcγ-receptors, but also other receptors may mediate important activating or inhibiting signals between effector cells and targets (Cartron et al. 2004).

COMPLEMENT DEPENDENT CELLULAR CYTOTOXICITY

Besides MAC formation and lysis of target cells, C activation also leads to opsonization of targets with C components C3b, inactivated C3b, and C4b. Complement activation products can be recognized by complement receptors expressed on effector cells. In this manner C activation can recruit cellular effector mechanism to destroy the C opsonized targets. In addition, the pro-inflammatory anaphylatoxins C5a and C3a stimulate inflammation and enhance the cytotoxicity of effector cells. CDCC must be taken into consideration, when studies concerning the effector mechanisms of antibodies are interpreted. If e.g. C activation is regulated at the C3 stage or one of classical C pathway components is depleted, along with direct C-mediated lysis also CDCC is blocked. Similarly, if the effector cells are depleted, the actions of both ADCC and CDCC are inhibited. In many studies where either CDC or ADCC have been studied, the role of CDCC has been underestimated or even ignored.

COMPARISON OF DIFFERENT EFFECTOR MECHANISMS

Elegant studies emphasizing either the role of C or of cellular mechanism as mediators of action of rituximab have been published. The interesting question arises: why do some results emphasize the role of ADCC and others that of CDC? It is possible that both mechanisms are important and that the lymphoma cells that manage to escape killing by one mechanism can still be vulnerable to attack by another mechanism. Support to this theory was obtained from recent studies by van Meerten et al. (van Meerten et al. 2006), who demonstrated that CDC and ADCC, even if acting independently, can significantly enhance killing of each other when combined. CD20-transfected CEM T-cells that were resistant to CDC were still sensitive to ADCC and vice versa.

In addition to combined action also different protocols used in the studies can explain the observed differences between CDC and ADCC. E.g. ADCC promoting experiments by Clynes (Clynes et al. 2000) were performed with a s.c. xenograft model in nude mice, whereas in studies supporting the role of CDC by Cregg and Di Gaetano tumor cells

were administered intravenously (Cragg, Glennie 2004, Di Gaetano et al. 2003). One can speculate that high levels of C in plasma may favor CDC in the context of i.v. administered tumors whereas rituximab killing in less vascularized tumors may require penetration of effector cells. On the other hand, Gong et al. demonstrated by using human CD20-transfected mice that the treatment with anti-CD20 antibody leads to a rapid clearance of circulating B-cells through the reticuloendothelial system (Gong et al. 2005). The elimination of circulating cells was strongly dependent on Fc-receptor related mechanisms, and the access of lymph node B cells to circulation was shown to be crucial for their depletion. In contrast, the elimination of B cells from the mantle zones, from where they are ineffectively mobilized, was demonstrated to be dependent on C activity.

In the introduction a cancer immunoediting theory concerning *de novo* oncogenesis was presented (Dunn, Koebel & Schreiber 2006, Dunn, Old & Schreiber 2004a, Dunn, Old & Schreiber 2004b). According to the theory the immune system can recognize and destroy the majority of transformed cells generated in the human body. However, few tumor cells are sometimes resistant to immune attack; they can survive and undergo slow transformation during subsequent years at the immunoediting phase. They often remain hidden without causing a clinical disease until new transformation or other events favoring the tumor cells lead to an escape from immune control. I hypothesize that analogous events may occur during therapeutic interventions. Immunochemotherapies have shown excellent primary response rates and the majority of patients have achieved at least a partial response. The overall response rates of immunochemotherapy in FL have been around 80 to 95% and complete response rates between 40 and 70%. The achievement of complete response has been shown to predict a longer progression free survival, but at least until recently, most patients with an initial complete response still tend to relapse during subsequent years. This obviously means that some tumor cells can resist the initial therapy, but that an intact immune system, and nowadays sometimes also maintenance therapy with rituximab, keeps them under control until new changes in gene expression lead to new escape mechanisms and cause clinical relapse. As could be expected, relapsed tumors are usually more resistant to rituximab and chemotherapeutics than the primary tumors, and even responding tumors tend to re-relapse earlier (Davis et al. 2000, Forstpointner et al. 2004, van Oers et al. 2006).

In animal studies cellular mechanisms have been shown to be important in the prevention of tumor development. In addition, the tumors developing in immunodeficient mice have been demonstrated to be more immunogenic than the tumors growing in wild type animals. This suggests that tumors must develop relatively efficient defence mechanisms against cellular immunity. In contrast significant differences in C regulator expression between different tumor types and individual tumors exist, but these differences do not seem to correlate with the behavior of the tumors. Our unpublished data failed to demonstrate difference in the incidence of chemically induced tumors between C deficient mice and WT mice. This suggests that the C system has only a minor role in the prevention of primary tumor development.

Fishelson has proposed that it may be in fact the absence of a selective force that keeps the C regulator levels variable (Fishelson et al. 2003). Complement regulators are constitutively expressed, and in the absence of effective C activating conditions the basal expression is sufficient to keep the tumors alive. As a result the growing tumors develop relatively effective defense mechanisms against cellular immunity, but not necessarily against C.

Another potential explanation for a less extensive C regulator expression on lymphoma cells arises from the special features of B cell development. During their maturation process, B cells are subjected to a series of positive and negative selection steps that control the naïve cell repertoire that has left the bone marrow. The selection is based on the exposure of B cells to self- and non-self structures at different stages of development. Negative selection leads to induction of apoptosis and deletion of autoreactive clones. The C system has been demonstrated to modify the selection processes and to participate in the clearance of apoptotic cells. To support C-mediated selection and to prevent the survival of autoreactive B cells, it can be speculated that it is beneficial that B cells do not express such a high amount of C regulators on their cell surfaces. This may explain why also malignant B cells express C regulators less extensively that many other tumor types.

The infusion of rituximab may be one of the first times when lymphomas are exposed to an effective C attack. Therefore it is not surprising that rituximab is effective in killing them. Rituximab-triggered C attack also provides the expected "selective force", which can enrich C resistant clones. As mentioned above tumor cells must have effective mechanisms to cope with cellular immunity to stay alive in body, so they may be indeed the differences in the C resistance that separate rituximab-responders from non-responders.

6.3 GENE EXPRESSION PROFILING AND THE ROLE OF TUMOR MICROENVIRONMENT IN THE PATHOGENESIS OF FL

Clinical results have demonstrated that despite the excellent primary responses, the clinical course of immunochemotherapy-treated patients varies significantly and the prediction of outcome of individual patients is difficult. Cytogenetic studies have confirmed that follicular lymphoma is a group of clonally heterogeneic diseases. Genetic differences are thought to affect the clinical diversity together with microenvironmental factors and additional genetic alterations that occur during the course of the disease.

Study IV searched for biomarkers related to the variable clinical course by evaluating gene expression profiles of lymphoma tissues of patients treated with R-CHOP. The idea was that this approach would provide a general overview of variability of biological features between lymphoma samples and would possibly reveal individual genes whose expression correlated to the outcome. In general, the expression patterns were relatively homogeneous, but certain genes with predictive power were identified. The genes

implicated in the long-term versus short-term outcome distinction included multiple regulators for cell growth, apoptosis, and immune responses. Interestingly, the genes whose expression was further validated with immunohistochemistry (EphA1, Smad1) were shown to be mainly expressed, not in the lymphoma cells themselves, but in the cells of the tumor microenvironment.

During the recent years several studies with analogous results have been published in follicular lymphoma. Dave et al. introduced an outcome predictor based on immune response-related gene expression signatures (Dave et al. 2004). A favorable prognosis profile included a number of genes strongly expressed by T cells and an unfavorable prognosis profile included genes related to dendritic cells and macrophages. In line with these results high macrophage counts were found to be associated with an aggressive disease (Alvaro et al. 2006b, Farinha et al. 2005), and high number of tumor infiltrating T-cells (Alvaro et al. 2006b), and especially of T-regulator cells (Carreras et al. 2006) with favorable prognosis. In our microarray data mRNA of the scavenger receptor on macrophages *marco* was overexpressed in the poor-prognosis patients. The expression of Marco in the tumor tissue may reflect a lymph node response to the tumor cells, because it has also been shown to be involved in tumor antigen stimulated phagocytosis by dendritic cells (Grolleau et al. 2003).

Glas et al. (Glas et al. 2005) could not verify the results by Dave, nor did they find any association between the macrophage count and outcome. Instead, they built their own 81-gene predictor, which could differentiate responders from non-responders, but could not predict the long-term outcome (Glas et al. 2005). The authors speculated that the outcome cannot be predicted using diagnostic samples because additional genetic mutations during the course of disease markedly affect the risk for transformation. This raises the question about the role of transformation for outcome. A consensus has been reached in that histologic transformation of FL to DLBCL is related to a worse prognosis, but early relapses are only sometimes related to transformation, and transformation can explain only a part of the diversity of the disease.

To explain the variable clinical behavior of FL with vulnerability of FL cells to transformation De Jong proposed a dual pathway model for the pathogenesis of FL (de Jong 2005). After the initial t(14;18) translocation different secondary alterations result in the development of prognostically different diseases. In the poor prognosis pathway, genomic alterations (Höglund et al. 2004) drive the process towards an activated immunologic state and a genomic instability. Follicular dendritic cells and tumor infiltrated T cells are thus highly activated, but they are dysfunctional with respect to homing of B cells. This results in an increased amount of interfollicular tumor cells, and an early vulnerability to the transformation of FL into DLBCL. In addition, the activation of T-helper 1 (Th1) cells may reflect immunologically active state and predict an early transformation (Glas et al. 2007). Alternatively, according to the theory of de Jong, in the good-prognosis pathway, genomic alterations accumulate at a slow pace

resulting in the stabilization of tumor cells. A mutagenic pressure from the microenvironment is lower, when follicular dendritic cells and T-cells, even if they are present at high density, are in an inactive state. Under these conditions the lymphoma cells are relatively resistant to transformation and the prognosis is better.

Besides the importance of C regulators and *marco*, our data revealed that the expression of Smad1 and EphA1 in non-malignant cells of tumor microenvironment was associated with a different outcome. Smad1 is a transcription factor related to BMP and TGF-β signaling and EphA1 is a receptor tyrosine kinase, which has been found to be involved in trans-endothelial migration of T-cells (Aasheim, Delabie & Finne 2005, Sharfe et al. 2002, Sharfe et al. 2003). EphA1 was detected in the vasculature and in granulocytes located close to high endothelial venules. The finding offered us a possibility to speculate that EphA1, which was overexpressed in the long-term-responders, may improve the ability of granulocytes to invade the malignant lymph nodes and to enhance rituximab-mediated ADCC.

The published molecular prognostic markers related to follicular lymphoma are summarized in Table 7.In the above mentioned microarray studies the treatment regimens were relatively variable and often not specified in detail by the authors, whereas our study focused on immunochemotherapy-treated patients. If we compare our results to the results of others, we can find both similarities and differences. Along with others we found outcome-associated genes related to the regulation of cell growth and immune responses, but also genes possibly related to the efficacy of rituximab-mediated CDC and ADCC (especially *CD55* and *epha1*). It is not surprising that these genes were not demonstrated in other studies where the treatment was not based on rituximab.

6.4 STRATEGIES TO IMPROVE EFFICIENCY OF MABS IN IMMUNOTHERAPY

Studies on the effector mechanisms of rituximab and on outcome-related biological factors have revealed tumor-related resistance mechanisms which lymphoma cells can use to escape immunotherapy. These findings establish a basis for strategies to improve the efficacy of the therapies. The strategies can be divided into (1) new methods to administer drugs, (2) improved selection criteria for patients, who are likely to benefit from immunochemotherapy, (3) methods to improve the response of target cells to rituximab, and (4) modifications to treatment regimens.

NOVEL METHODS TO ADMINISTER RITUXIMAB

The results in this work have shown that intravenously administered rituximab does not enter the central nervous system. Low levels of rituximab in CSF cannot reduce the amount of CD20 positive cells in the CSF nor induce any therapeutic effect. We have suggested that intrathecal administration of rituximab might be a tempting alternative for patients with lymphoma involving the CNS (II). Recently this approach was tested by others. Intrathecal rituximab has been shown to be effective against leptomeningeal

lymphoma, but ineffective against parenchymal disease (Schulz et al. 2004). However, Takami and his colleagues injected intraventricularly rituximab and autologous serum as a source of C to one patient (Takami et al. 2006). They demonstrated a late clinical response against parenchymal CNS lymphoma, but because of infection problems only one infusion was given, and the disease soon progressed.

Table 7. The published molecular prognostic markers related to follicular lymphoma. Also predictive factor related to immune system reactions are included in table.

Factor	*Mechanism*	*Effect*	*Reference*
BCL-2 expression	Anti-apoptotic	Unfavorable	
BCL-6 expression	Germinal center phenotype	Favorable	Bilalovic (Bilalovic et al. 2004)
CD10 expression	Germinal center phenotype	Favorable	Bilalovic (Bilalovic et al. 2004)
Bcl-XL	Anti-apoptotic	Unfavorable	Zhao et al. 2004)
Cyclin B1	Cell cycle regulation	Favorable	Björck et al. 2005
Epha1	Migration	Favorable	Paper IV
Smad1	Signal transduction	Unfavorable	Paper IV
Marco	Macrophage receptor	Unfavorable	Paper IV
CD55	Regulator of C activation	Unfavorable	Paper III
Fcγ-receptor polymorphisms	Regulation of ADCC	Variable	Cartron et al. 2002
Activated follicular dendritic cell network	Rapid transformation to DLBCL	Unfavorable	de Jong 2005
Activation of T-helper 1		Unfavorable	Glas et al. 2007
Treg count		Favorable	Carreras et al. 2006
Macrophage count		Unfavorable	Alvaro et al. 2006b, Farinha et al. 2005
Immune response 1 (IR-1) versus IR-2		Variable	Dave et al. 2004
81-gene predictor		Variable	Glas et al. 2005

IMPROVED PATIENT SELECTION

To identify beforehand the patients to whom immunochemotherapy most probably is not effective, more accurate methods to select patients are needed. As discussed above clinical prognostic indices does not provide any information about tumors. Desired valuable molecular information on lymphomas can be received by gene expression profiling but it cannot be incorporated into routine diagnostics or clinical evaluation of patients. A better approach would be to use less laborious and costly methods, such as flow cytometry, immunohistochemistry, or qPCR. The latter two approaches have been

utilized in recent studies on diffuse large B-cell lymphoma (Berglund et al. 2005, Hans et al. 2004). In this work we have used flow cytometry, qPCR and immunohistochemistry for validation of microarray data, and demonstrated that mRNA analyses can be extended to protein studies. We have also presented some candidate proteins, which might discriminate long surviving patients from those whose disease relapses early. If future studies with larger patient cohorts confirmed our results, pre-treatment analysis of these parameters might provide a practical tool for the selection of patients or for the prediction of outcome. By now none of the recently discovered molecular predictors has been incorporated into widespread clinical use, but most probably in near future prognostic indices based on the molecular markers, or indices combining clinical and molecular factors will be proposed.

MECHANISMS OF RESISTANCE TO RITUXIMAB

A great number of NHL patients treated with rituximab monotherapy become resistant to rituximab. In a re-treatment study of patients with relapsed follicular or low-grade NHL, only 40% of patients who had a prior partial or complete response to single-agent rituximab responded to re-treatment at the time of relapse (Davis et al. 2000). The mechanism of resistance is not clearly defined, but most probably the development of resistance is a result of multiple events. The discovery of factors influencing in the efficacy of rituximab treatment has also revealed tumor escape mechanisms utilized by lymphomas. These factors can be categorized as (1) factors influencing the number of circulating CD20-positive cells or the level of CD20 expression, (2) C resistance mechanisms, (3) ways to affect ADCC, and (4) ways to modify CD20-mediated signaling.

The amount of CD20 molecules

Approaches to increase CD20 expression on B-cells are limited, but in one study CD20 expression was shown to be increased after treatment with TNFα, GM-CSF and IL-4 (Venugopal et al. 2000), and in another study after treatment with bryostatin-1 (through the ERK signal transduction pathway) (Wojciechowski et al. 2005). Clinical studies with these agents have not been published, so their clinical impact remains to be shown.

Kennedy et al. have demonstrated that rituximab-opsonized CLL cells were temporarily removed from the circulation by the mononuclear phagocytic system, and released back after the rituximab-CD20 complexes were removed by phagocytic cells (Kennedy et al. 2004). They proposed that this escape mechanism would produce CLL cells that lack CD20 molecules and simultaneously could explain the loss of rituximab sensitivity observed in some CLLs. To overcome this escape mechanism the authors suggested that it might be worth of engineering a modification of anti-CD20 antibody, which would activate C but not bind to Fc-receptors. However, this kind of modification would also loose the ADCC inducing capability of rituximab.

Enhancement of C activity

Complement-activating potential of mAbs with different mouse isotypes varies considerably. Isotype switching, humanization and other types of manipulation may be used to strengthen the C activating power of antibodies. Since rituximab already is a chimeric mAb of the C activating human IgG1 isotype, these procedures are no longer practical.

Neutralization of C regulatory molecules could be a useful method to enhance rituximab-mediated CDC also *in vivo*. Because also all normal cells need C regulatory molecules on their cell surfaces, the neutralization should be directed specifically to lymphomas. This could be achieved by generating bispecific or cross-linked antibodies, which would recognize a tumor-specific antigen and a C regulator molecule (Blok et al. 1998). The potentially harmful effects of antibody binding to normal tissues could be prevented by composing constructs with a high-affinity tumor-detection arm (or antibody) and a low-affinity C regulator-detection arm (Gelderman et al. 2002, Harris et al. 1997). Another approach would be to modulate C regulator expression with cytokines, but as mentioned earlier many cytokines have usually been demonstrated to have rather an up-regulative than a down-regulative effect. Interestingly, however, fludarabine treatment has been shown to down-regulate CD55-expression levels in B lymphoma cells. It has also made lymphoma cells more vulnerable to lysis by rituximab (Di Gaetano et al. 2001). The recently described siRNA technology (Elbashir et al. 2001) provides yet another method to block the expression of C regulators. Terui and colleagues knocked-down CD55 expression from freshly isolated lymphoma cell lines with siRNA (Terui et al. 2006) and received cells, which were more vulnerable to rituximab treatment. Nagajothi demonstrated genetic and biochemical methods to decrease the expression of GPI-anchored proteins (Nagajothi et al. 2004), but studies in the context of rituximab have not been published.

Kennedy and his colleagues have suggested that deficiencies in one or more C components may be associated with a failure of some patients to achieve effective response to rituximab (Kennedy et al. 2004). They observed that the C component C2 was sometimes consumed out during rituximab treatment of CLL with high cell densities, and suggested that this might explain the poor response of some patients to rituximab. As a solution they proposed to use additional C2 or a more universal C source, like plasma infusion together with rituximab (Kennedy et al. 2004).

CD20 expression levels have been demonstrated to have a sigmoidal correlation with CDC (van Meerten et al. 2006). This suggests that the induction of effective C activation requires a certain minimum level of CD20 on cell surfaces. High density of CD20 molecules leads to stronger binding of antibody and C1q molecules and enables classical pathway activation to proceed efficiently to terminal pathway activation. In an attempt to improve complement activation after rituximab treatment, an antibody directed to iC3b

was added to rituximab and complement-treated tumor cells (Kennedy et al. 2003). As a result more C3b molecules accumulated onto the tumor cell surfaces and the cells were killed more efficiently by complement.

Enhancement of ADCC activity

The precise contribution of various effector cells to rituximab-mediated ADCC remains unclear but at least NK-cells and monocytes, and in some studies also PMNs, have been demonstrated to contribute to ADCC activity. Several cytokines have potential to increase the number of different types of leukocytes and they have been proposed to enhance ADCC activity. In animal studies IL-2 has been shown to promote NK-cell development and activation (Hooijberg et al. 1995). Thereby it may enhance also rituximab activity. When IL-2 was given together with rituximab, a positive correlation between high NK cell count and a favorable clinical response was found (Gluck et al. 2004). Clinical trials have also shown increased progression free survivals for patients treated with rituximab and IFN-α (Kimby 2002). Also granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) may enhance the cytotoxic activity of effector cells in ADCC (Kimby 2002, Voso et al. 2002). We used leukocyte stimulators PMA or FMLP to stimulate peripheral blood leukocytes *in vitro*, but no effect on rituximab-triggered ADCC was observed. However, the promising clinical results of combining IFN-α to rituximab therapy and the desire to avoid the use of chemotherapeutics have aroused interest to study further the efficacy of cytokine-adjusted rituximab therapies. Also adoptive transfer of LAK cells was demonstrated to improve the rituximab-mediated ADCC, but this study only had seven patients (Berdeja et al. 2007).

The interaction of antibody with Fcγ-receptors of effector cells is a fundamental step in the triggering of ADCC. In several studies the balance between activating (CD16, CD32 (Fcγ-RIIa) and CD64) and inhibiting (CD32 - Fcγ-RIIb) Fc-receptors has been investigated and a correlation to the efficacy of ADCC has been observed (Weng, Levy 2003). Therefore approaches to influence this balance or molecular engineering of the Fc region of rituximab to increase its affinity to Fc-receptors may be effective in enhancing ADCC (Shields et al. 2001).

Altered signaling

Non-toxic sensitizing agents (e.g. drugs, chemotherapeutics, rituximab) alter gene expression profiles by regulating the expression levels of anti- and pro-apoptotic proteins (Jazirehi et al. 2001, Jazirehi, Bonavida 2005, Maldonado, Melendez-Zajgla & Ortega 1997). The use of such agents could remove inhibition blocks of apoptosis or lower the threshold to apoptosis and may be used prior to the proper cytotoxic therapy. According to a model proposed by Jazirehi (Jazirehi, Bonavida 2005) cytotoxic secondary agents can thereafter destroy the targets at lower concentrations and with fewer side effects.

ALTERNATIVE METHODS TO TREAT IMMUNOCHEMOTHERAPY RESISTANT PATIENTS

By now, if immunochemotherapy has proven not to be effective, the only alternative has often been to change treatment regimen. Currently available approaches for these situations include use of rituximab with other chemotherapeutics, autologous stem cell transplantation, maintenance therapy, and radioimmunotherapy.

Stem cell transplantation and maintenance therapy

When active treatment of relapses is indicated, intensive treatment i.e. consolidation high-dose chemotherapy with autologous (HDT/ASCT) or allogeneic stem cell transplant is worth considering. Randomized studies have shown that HDT/ASCT prolongs the progression-free survival (Freedman et al. 1999, Lenz et al. 2004, Schouten et al. 2003) of chemotherapy-treated patients, but that initial treatment with immunochemotherapy gives a similar effect. Intensive treatment is considered in Finland for early relapsed patients with high FLIPI scores (≥3) and for patients with transformed disease (Jyrkkiö et al. 2007). Also allogeneic hematopoietic stem cell transplantation is a potentially curative treatment, but treatment-related mortality is higher compared to autologous transplantation (van Besien et al. 2003). Allogeneic transplantation may be considered for young patients relapsed after ASCT.

Not all patients are suitable for intensive treatment, but FL is also a good candidate for maintenance therapy. Maintenance treatment with rituximab has given survival benefit for patients primarily treated with either chemotherapy or immunochemotherapy (Forstpointner et al. 2006, van Oers et al. 2006) but not for rituximab-resistant patients.

Radioimmunotherapy in follicular lymphoma

In addition to rituximab, radiolabeled non-humanized anti-CD20 antibodies ^{90}Yttrium-ibritumomab and ^{131}Iodine-tositumomab have been approved for treatment of FL. The major problem with these agents is a two to four weeks lasting myelosuppression. It is usually observed one to two months after radioimmunotherapy, but can be controlled with dose escalation. In a phase III study of relapsed follicular and other indolent NHLs ^{90}Yttrium-ibritumomab was compared to rituximab (Witzig et al. 2002). The overall response rate was higher for Y-ibritumomab (80% versus 56%), but there was no significant difference in times to progression. ^{131}Iodine-tositumomab has been compared to chemotherapy in clinical trials with superior response rates (Kaminski et al. 2001, Press et al. 2003). No trials comparing radioimmunotherapy to immunochemotherapy have been conducted. In Finland, the use of radioimmunotherapy has by now been limited to special cases.

6.5 FUTURE PROSPECTS

Specific immunotherapies have started a new era in cancer treatment. The research field of immunotherapy is young and we are still drawing the rough lines and characterizing the basic features. The research is still giving more clues than answers. In this thesis the effector mechanism of rituximab, and factors related to its efficacy in the treatment of

FL patients have been investigated. We have demonstrated novel molecular markers (EphA1, Smad1, Marco, CD55) whose expression correlate to clinical course of immunochemotherapy treated patients, and emphasized the role C as an important effector mechanism of rituximab. This indicates that more attention needs to be paid to the properties of monoclonal antibodies. Modification and engineering of antibody molecules provide practical tools for effective and specific cancer therapy.

After rituximab several other monoclonal antibodies have already been launched for cancer therapy and many more are coming. Making the study of rituximab even more interesting the experience we receive from rituximab can be utilized also in the development of novel antibodies in the future. It is probable that the mechanisms of action of rituximab and at least some of the obstacles we are facing with rituximab therapy are more universal, and can be extended as general principles related to monoclonal antibody therapy.

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