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Immunotherapy with the Anti-EpCAM Monoclonal Antibody and Cytokines in Patients with Colorectal Cancer

A Clinical and Experimental Study

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This work is dedicated
To all patients who have
participated in this
clinical study
and
To my family

ABSTRACT

The tumor-associated antigen EpCAM (epithelial cell-adhesion molecule) (CO17-1A) is over expressed by various human carcinomas, including colorectal carcinoma (CRC). This antigen can be used as a target structure for specific immunotherapy with vaccines and monoclonal antibodies (MAb). Addition of cytokines to MAb therapy may augment immune effector functions and chemotherapeutic agents may also add to therapeutic efficacy.

In this thesis, we have analysed clinical and immunological responses of patients with advanced CRC treated with either the murine anti-EpCAM MAb (anti-EpCAM mMAb) or its chimeric counterpart (anti-EpCAM cMAb) in combination with cytokines and chemotherapeutics. Additionally, sequential analysis of cytokeratin positive (CK+) cells in the bone marrow (BM) were made in CRC patients receiving MAb based therapy for advanced disease or as adjuvant therapy.

Pretreatment natural killer (NK) cell cytotoxicity in vitro of peripheral blood mononuclear cells was an independent prognostic factor for overall survival and progression free survival (PFS) in patients receiving anti-EpCAM MAb based therapy as first-line therapy. The results from this study might be used for future patient selection and indicate that agents that activate NK cells should be considered to MAb-based treatment regimens.

The addition of GM-CSF, α-interferon and 5-fluorouracil to anti-EpCAM mMAb seemed to improved the antitumor response rate compared to historical control patients treated with anti-EpCAM mMAb alone (54% *v*s 15%) as well as PFS (15 *vs* 7 weeks). Clinical effects were mainly stable disease > 3months (11 of 14 responders) and responding patients survived longer than non-responders. The clinical efficacy of anti-EpCAM cMAb and GM-CSF was not better than in a historical control group who had received the anti-EpCAM mMAb and GM-CSF (overall response rates=21% *vs* 27%, respectively). Anti-idiotypic antibody (Ab2) concentrations as well as the frequency of patients mounting an Ab2-response in anti-EpCAM cMAb treated patients were lower as compared to anti-EpCAM mMAb-treated patients (69% *vs* 100%).

Following repeated daily subcutaneous (s.c.) injections of exogenous non-glycosylated *E.coli*-derived GM-CSF (molgramostim), the peak serum GM-CSF concentrations declined days 5 and 10 as compared to day 1. A dose-dependent increment in total white blood cell count was observed, the total numbers of GM-CSF receptor expressing cells increased during treatment while a transient decline in expression intensity was observed at day 5. The majority of patients developed binding but not neutralizing anti-GM-CSF antibodies. These results might support a receptor-mediated clearance of GM-CSF from the circulation. Importantly, high dose of GM-CSF resulted in lower antibody-dependent cellular cytotoxicity that may reflect immune suppression. Further studies are required to establish the optimal biological dose of different cytokines.

CK+ cells in BM were examined by immunohistochemistry on routinely processed BM clots, and CK+ cells were divided into different subtypes; Group A (CK+ probably malignant epithelial cells), Group B (CK+ morphologically non-epithelial cells) and Group C (CK+ contaminating cells). The presence of Group A cells did not adversely affect the prognosis while the presence of Group B cells probably indicates a poor prognosis in patients receiving adjuvant therapy. Sequential BM aspirations do not seem to add to the existing methods to follow the effect of treatment in CRC.

These results might provide further clinical studies with MAbs, combined with other agents with different modes of action to increase the clinical efficacy of MAb. Ideally, patients with a well preserved immune system and low or minimal tumor burden should be selected to MAb-based therapy.

Key words*:* Colorectal carcinoma, monoclonal antibodies, GM-CSF, pharmacokinetics, prognosis, bone marrow micrometastases, cytokeratin-positive cells

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LIST OF PUBLICATIONS

The thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. **Liljefors M**, Nilsson B, Hjelm Skog A-L, Ragnhammar P, Mellstedt H, Frödin J-E: Natural killer (NK) cell function is a strong prognostic factor in colorectal carcinoma patients treated with the monoclonal antibody 17-1A. *Int J Cancer* 105:717-723 (2003).

II. **Liljefors M**, Ragnhammar P, Nilsson B, Ullenhag G, Mellstedt H, Frödin J-E: Anti-EpCAM monoclonal antibody (MAb 17-1A) based treatment combined with α-interferon, 5-fluorouracil and granulocyte-macrophage colony-stimulating factor in patients with metastatic colorectal carcinoma. *Int J Oncology* 25:703-711 (2004).

III. **Liljefors M**, Nilsson B, Fagerberg J, Ragnhammar P, Mellstedt H, Frödin J-E: Clinical effects of a chimeric anti-EpCAM monoclonal antibody in combination with granulocyte-macrophage colony-stimulating factor in patients with metastatic colorectal carcinoma. *Int J Oncology* 26:1581-1589 (2005).

IV. **Liljefors M**, Nilsson B, Mellstedt H, Frödin J-E: Influence of varying doses of granulocyte-macrophage colony-stimulating factor on pharmacokinetics, hematological and immune functions. Manuscript 2005.

V. Shetye J, **Liljefors M**, Emdin S, Frödin J-E, Strigård K, Mellstedt H, Porwit A: Spectrum of cytokeratin-positive cells in the bone marrows of colorectal carcinoma patients. *Anticancer Res* 24;2375-2384 (2004).

VI. **Liljefors M**, Frödin J-E, Porwit A, Ragnhammar P, Mellstedt H, Shetye J: Repeated bone marrow analyses for cytokeratin positive cells in patients treated for colorectal carcinoma. Manuscript 2005.

ABBREVIATIONS AND GLOSSARY

Ab	Antibody
Ab1	Idiotype
Ab2	Anti-idiotypic antibody
Ab3	Anti-anti-idiotypic antibody
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
AJCC	American Joint Committee on Cancer
anti-Id	Anti-idiotypic antibody
Anti-anti-Id	Anti-anti-idiotypic antibody
APC	Antigen presenting cell
BM	Bone marrow
BMA	Bone marrow aspiration
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CK	Cytokeratin
cMAb	Chimeric monoclonal antibody
CR	Complete remission
CRC	Colorectal carcinoma
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic lymphocyte antigen 4
CSF	Colony stimulating factor
Da	Dalton
DC	Dendritic cell
DTH	Delayed type hypersensitivity
ELISA	Enzyme linked immunosorbent assay
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell-adhesion molecule
F(ab)	Fragment antigen binding
FasL	Fas ligand
Fc	Fragment crystalizable
FcγR	Fc gamma receptor
FR	Framework region
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HACA	Human-anti-chimeric antibody
HAHA	Human-anti-human-antibody
HAMA	Human-anti-mouse-antibody
huMAb	Humanized monoclonal antibody
HLA	Human leukocyte antigen
IFN	Interferon

Id	Idiotype
Ig	Immunoglobulin
IL	Interleukin
i.v.	Intravenous
κ	Kappa
λ	Lambda
MAb	Monoclonal antibody
mMAb	Mouse monoclonal antibody
MHC	Major histocompatibility antigen
MR	Minor response
MRD	Minimal residual disease
MTD	Maximum tolerated dose
NK	Natural killer cell
ORR	Overall response rate
OS	Overall survival
OTD	Optimal therapeutic dose
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PFS	Progression free survival
PD	Progressive disease
rh	Recombinant human
s.c.	Subcutaneous
SD	Stable disease
TA	Tumor antigen
TAA	Tumor associated antigen
TAM	Tumor infiltrating macrophage
Tc	T cytotoxic
TCR	T cell receptor
Th	T helper
TIL	Tumor infiltrating lymphocyte
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TSA	Tumor specific antigen
VEGF	Vascular endothelial growth factor
VH	Variable heavy chain
VL	Variable light chain
WBC	White blood cell

Antigen; A molecule that can be recognized by the adaptive immune system.

Epitope (antigen determinant); A specific part of an antigen recognized by an immune receptor.

Idiotope (individual idiotypic determinant); An antigenic determinant of an immunoglobulin molecule or T cell receptor that is constituted from the variable region of the molecule.

Idiotype; Collection of idiotops on an individual immune receptor.

Ligand; The antigen to which the antibody binds to.

Affinity; Is the binding strength between the antibody and its epitope. It is the sum of cohesive and repulsive forces.

Avidity; Is the overall strength of binding of an antibody molecule to an antigen or particle. Antibodies have more than one antigen binding site and larger antigens (bacteria or viruses) have multiple identical epitopes that the antibody can bind to.

TABLE OF CONTENTS

1 THE IMMUNE SYSTEM

The human immune system is a complex network of molecules, cells and organs that protect the host from damage caused by invading microorganisms, such as bacteria, viruses, fungi and parasites, or own altered internal cells.

The origin of immunology is usually attributed to Edward Jenner (1749–1823) who discovered the successful protection of humans against smallpox infection by vaccination with cowpox or vaccinia virus. The term vaccination is still used to describe the same procedure as Jenner introduced: inoculation with a dead or attenuated form of the microorganism to provide protection from disease.

Principally, there are two types of immune responses: the innate system and adaptive system, with extensive crosstalk between the two. There are distinctive differences in the mechanisms and receptors used for the immune recognition between innate and adaptive immunity [1].

1.1 Innate immunity

The innate immune system exerts its effect independently of prior contact with a pathogen and include cellular and molecular components that react quickly (within minutes) to molecular patterns found in microbes.

Innate immune recognition is mediated by highly conserved structures present in large group of microorganisms. These structures are referred to as pathogen-associated molecular patterns (PAMP), and the receptors of the innate immune system that used to recognize them are called pattern-recognition receptors (PRR). Examples of PAMP are bacterial lipopolysaccharide, mannans and bacterial DNA.

Surface epithelial barriers (skin, surface of mucous membrane) and *physiological barriers* (pH, temperature) serve important innate immune functions by preventing the entry of microbes.

Soluble factors such as complement, acute-phase proteins and cytokines are parts of this system. *Complement* is a complex of plasma proteins, that can be activated by one of three pathways [2], the classical pathway is activated following binding of antibodies (immunoglobulin; see section 1.2.1) to the pathogen. The lectin and alternative pathways are generally activated by pathogens and not by antigen-antibody complexes. The outcome of complement activation generates a number of immunological active substances and deposits of complement + fragments on the surface of pathogens. This event enhances phagocytosis of the pathogen and release of inflammatory mediators by effector cells of the innate system [3].

Cytokines (“cell movers”) constitute another group of soluble mediators in the innate immune system. They are glycoproteins which are active in picomolar to nanomolar concentrations and play an important role as messengers between the

innate and adaptive immune systems. Furthermore, they are involved in the regulation of inflammation in both physiological and pathological condition and in haematopoesis [4]. The main groups of cytokines are interferons (IFN) interleukins (IL) and colony-stimulating factors (CSF) (G-CSF, M-CSF, GM-CSF) (section 2.1.2). The IFN group consists of three types: IFNα, IFNβ and IFNγ. IFNs make several contributions to defense against viral infection by the induction of a state of resistance to viral replication.

Unlike hormones, which are secreted by one type of cell, most cytokines are produced by a broad range of cells, i.e. macrophages, T lymphocytes, endothelial cells and stroma cells of the bone marrow, and affect the growth and metabolism of a wide array of cells through autocrine and paracrine pathways activities.

Cells, such as *macrophages* (derived from blood-borne monocytes) and *granulocytes* (Figure 1) can release high concentrations of toxic molecules like nitric oxide, lysozyme which can destroy adjacent cells. Furthermore, they can phagocytose microorganisms and also the body's own dead or dying cells.

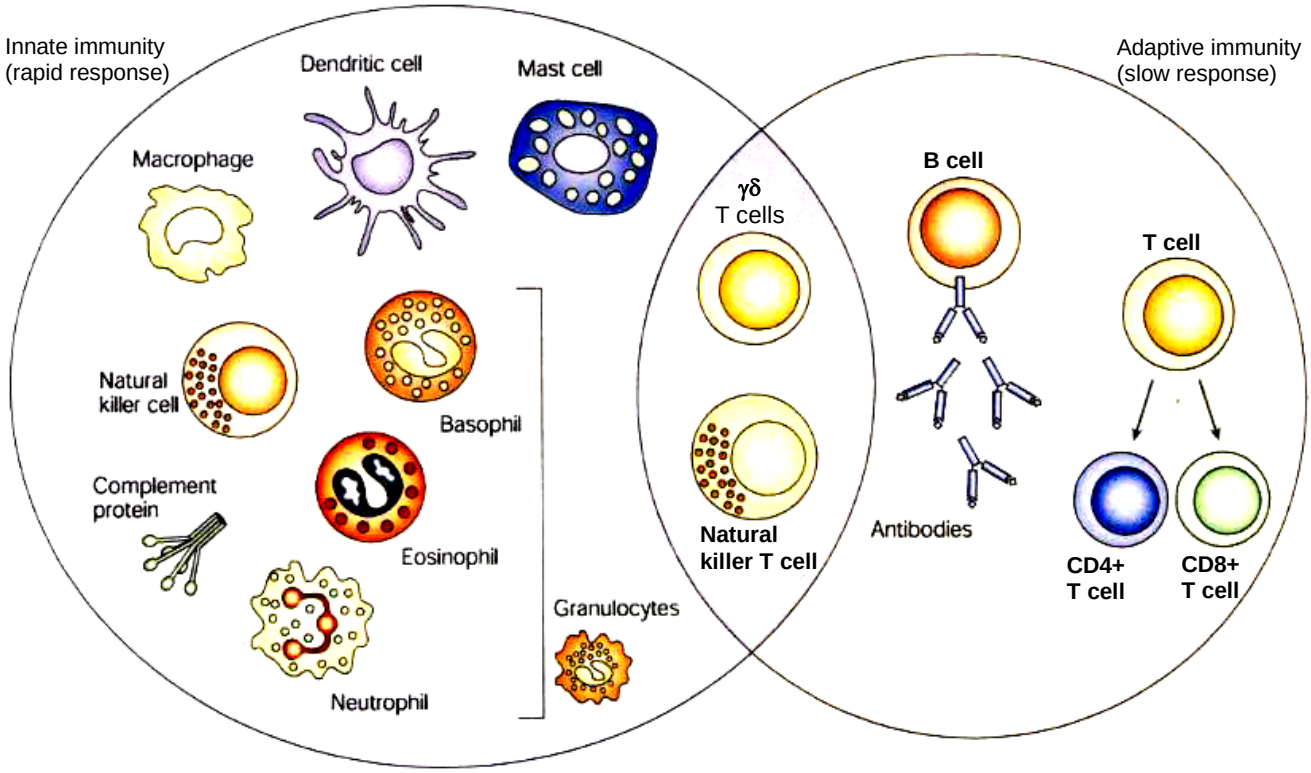

Figure 1. The innate and adaptive immune response. The innate immune response functions as the first line of defence against infection. It consist of *soluble factors*, such as complement proteins, and *cellular components*, including granulocytes, mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response consists of B cells and T cells. γδ T cells and natural killer T cells are cytotoxic lymphocytes that function at the intersection of innate and adaptive immunity. (Reproduced with permission from Nature Reviews Cancer Vol. 4, pp 11-22 © (2004), Macmillan Magazines Ltd).

Natural killer (NK) cells are bone-marrow derived lymphocytes capable of mediating early innate immune responses to virally infected cells and transformed cells (section 1.6). They comprise approximately 5%–20% of peripheral blood lymphocytes but contrary to lymphocytes of the adaptive immune system, they lack surface molecules necessary for specific recognition of antigens [5]. Like other effector cells of innate immunity, activated NK cells produces cytokines such as IFNγ, GM-CSF, TFNα and others thereby activate and regulate other immune components. Besides NK cells, interdigitating *dendritic cells (DCs)* comprise essential links between innate and adaptive immunity [3]. DCs are a heterogeneous group of bone-marrow derived leukocytes that display differences in anatomic localization, cell surface phenotype, and function [6], distributed throughout the body. Immature DCs constantly phagocytose, endocytose and process extra cellular antigens. They begin to mature after the stimulation of maturation inducing signals as “danger signals” (bacteria or their products, lipopolysaccarides, viruses, inflammatory cytokines) or binding of pathogen products to PRR, such as toll-like receptors [7]. DCs are suggested to be the most important type of antigen presenting cells (APCs) that stimulate T lymphocytes and play a main role in the induction of an immune response [6,8].

1.2 Adaptive immunity

Unlike the innate immunity, adaptive (acquired) immune responses are specific for the inducing agent, and is marked by an enhanced response on repeated encounters with that antigen. Thus, the key features of the adaptive immune response are *specificity* and *immunological memory*. The adaptive immune system responds to a pathogen only after it has been recognised by the innate immune system [1,9].

The adaptive immunity is composed of two lymphocyte subpopulations; B cells (B cells were called that because they were first identified in the “Bursa of Fabricius” in chicken) constitute the humoral immunity and T cells (Thymus-derived) constitute the cellular immunity.

1.2.1 B cells and antibodies

In 1898, a German bacterologist Paul Ehrlich (1854–1915) postulated the concept that immune cells can secrete receptors (“side-chains”) in response to invasive particles and transformed cells. Ehrlich prophesied their use as “Magic Bullets” [10]. Today we know that these “side-chains” receptors represent antibodies, which are antigen-specific immunoglobulins (Ig), responsible for the rapid elimination of extracellular and mucosal microorganisms, produced by B-cells. Every B cell has numerous Ig molecules on its surface that recognises a unique three dimensional antigenic determinant (epitope) [9].

All Ig molecules have a similar basic structure unit comprised of two identical heavy chains and two identical light chains coupled together with disulphide bridges. In any given Ig molecule, the two heavy chains and the two light chains are identical, giving an antibody molecule two identical antigen-binding sites (Figure 2) and thus the ability to bind simultaneously to two identical structures. The light chains refer to lambda (λ) and kappa (κ). There are five main heavy chain classes, or isotypes (IgM, IgD, IgG, IgA, IgE) and these determine the functional activity of an antibody molecule. In humans, IgG can be further divided into four subclasses (IgG1, IgG2, IgG3, IgG4) whereas IgA antibodies are found as two subclasses (IgA1 and IgA2). IgG is by far the most abundant Ig and constitutes about 80% of the antibodies in serum.

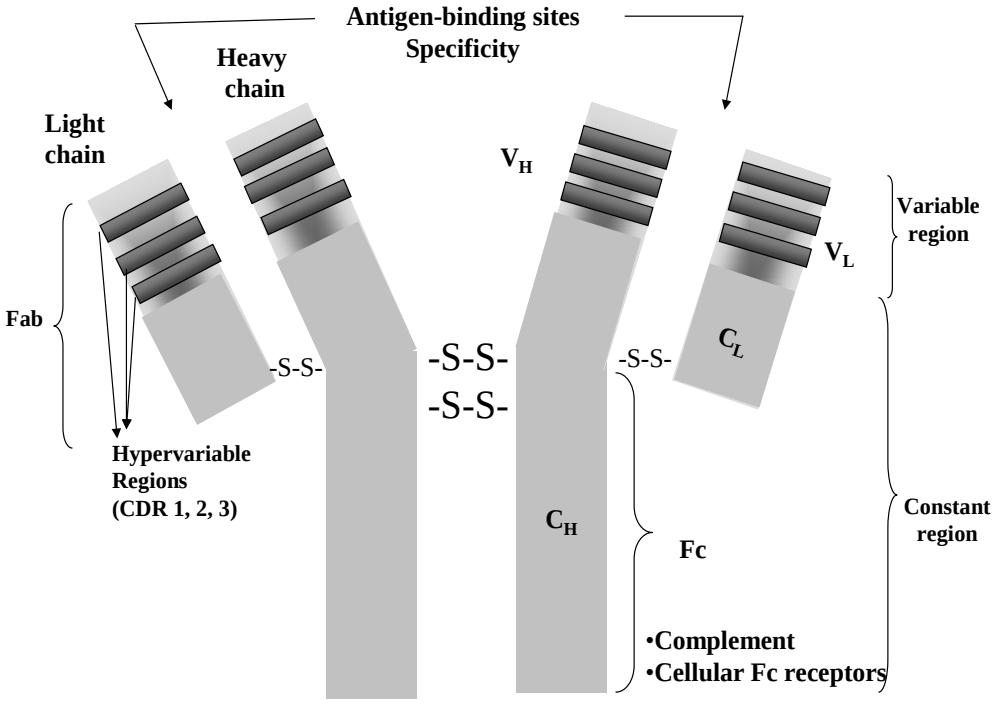

Figure 2. Basic structure of an IgG molecule. The amino-terminal variable domains (V domains) of heavy and light chains (V_H and V_L) together make up the V region of the antibody and confer on it the ability to bind specific antigen whereas the constant domains (C domains) of the heavy and light chains (C_H and C_L, repectively) make of the C region. Within the V-regions, there are hyper variable, complementarity determining regions (CDRs), which bind to the antigen and determine antigen specificity. –S-S-=disulphide bridges. Pepsin cleaves the lg molecule in one F $(Ab')_2$ fragment with the same antigen-binding characteristics as the original antibody and can be used for therapy [9]. Papain cleaves the lg molecule in three pieces; two identical Fragment antigen binding (Fab) fragments containing the V regions, and one Fragment crystallizable (Fc) fragment containing C regions without antigen binding activity.

Each type of antibody can be produced as a circulating molecule or as a stationary molecule. The stationary antibody functions as the B cell receptor. If a naïve B cell (one that has not previously encountered its specific antigen) finds an epitope that matches its specific Ig molecule, the cell starts to divide rapidly, and differentiates into memory B cells and/or antibody secreting plasma cells. The circulating antibodies can bind to the epitope of microorganisms or transformed cells and destroy them either directly or by activating the complement system or induce the antibody dependent cellular cytotoxicity (ADCC) (section 2.2.2).

The function of an antibody, to bind an antigen, is accomplished by the interaction between the variable (V) regions and the antigen. Both the heavy chain and light chains have V-regions and constant (C) regions (Figure 2). Within the V-regions, there are hypervariable, complimentary determining regions (CDRs), which bind to the antigen and determine antigen specificity. The regions between the CDRs are named framework (FR) regions and show less variability. Of importance, the functional differences between heavy-chain isotypes lies mainly in the Fc fragment.

The Fc portions of antibodies of certain isotypes are recognised by specialised receptors (Fc-receptors) expressed by immune effector cells. The Fc portions of IgG1 and IgG3 antibodies are recognised by specific Fcγ receptors (FcγRs) present on the phagocytic cells such as macrophages and neutrophils which can bind and engulf pathogens coated with antibodies of these isotypes. For IgG three different classes of FcγRs have been characterized: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16).

1.2.2 T cells

Precursors for T cells migrate from the bone marrow and mature in the thymus. During thymic maturation, thymocyte start to express a unique antigen-binding molecule, called the T cell receptor (TCR). In contrast to the immunoglobulins, that recognize pathogens in the extra cellular spaces of the body, the T cells with their TCR is restricted to recognize only short amino acid chains (peptides) displayed on the surface of the body's own cells in conjunction with cellmembrane-proteins called major histocompatibility complex (MHC). To ensure the development of mature MHC-restricted T cells that can discriminate between self and non-self antigens, thymocytes undergo a process named thymic selection (involving positive and negative selection) during the T cell development [3,9].

The TCR consists of α/β heterodimers, expressed in the cell membrane in association with a signaling unit termed CD3. A minority of T cells bear an alternative receptor made up of γ/δ heterodimers.

There are two main types of T cells; helper T cells (Th)(CD4+) and cytotoxic T cells (Tc)(CD8+). CD4 and CD8 are membrane glycoproteins on their surface and serve as co-receptors for delivering TCR/CD3 signals. CD4+ T cells can be further

divided into T helper 1 (Th1) and Th2 cells based on their cytokine secretion profile: Th1 cells secrete IL-2 and IFNγ, to activate NK cells, macrophages and to prime and maintain an antigen specific CD8+ T cell response (cytotoxic T lymphocytes (CTL), thus favour a cell-mediated immune response.

Th2 cells secrete IL-4,5,6,10 and 13 which promotes activation and differentiation of B cells, thus favour a predominantly humoral response. Additionally, CD4+ T cells might also have a cytotoxic activity [11,12].

In addition, several types of regulatory T cells have been described (section 1.7).

1.3 Antigen presentation and recognition for T cells

There are two major types of MHCs, class I and II. MHC class I molecules consist of a membrane-inserted heavy chain and a smaller non-covalenty associated chain, β2-microglobulin (β2m). MHC class II molecules consist of a non-covalent complex of two chains, α and β. In humans, MHC class I molecules correspond to the human leukocyte antigen (HLA)-A, -B and -C molecules, and MHC class II molecules correspond to HLA-D molecules. The amino acids recognised by the TCR derive from both the MHC molecule and the antigenic peptide. Thus, the TCR recognises an individual's own MHC molecules (self) together with peptides from foreign antigens.

MHC class I molecules are expressed on all nucleated cells and in general present 8-11 amino acid-long peptides from endogenous antigens. These antigens include viral proteins and unique proteins associated with the ontogeny of cancer cells. The MHC class I-peptide complex is recognised by CD8+ T cells (Figure 3). Following this recognition, the CD8+ cell undergoes clonal expansion and differentiates into mature CD8+ and memory CD8+ cells. Mature CD8+ T cells move through the body, searching for cells that possess complexes to which the TCR will bind and proceed to destroy those cells. Memory CD8+ T cells function like memory B cells, they persist and will multiply if they are re-exposed to the same MHC complex.

MHC class II molecules are mainly expressed on the surface of "professional" antigen-presenting cells (APC); a group that includes B lymphocytes, macrophages and especially DCs. DCs are supposed to be one of the most important cell types for initiating the priming of naïve CD4+ helper cells and for inducing CD8+ T cell differentiation into killer cells [6]. As a general rule, exogenous antigens enter into the MHC class II processing pathway and presented as 9–31 amino acid-long peptides to CD4+ T cells (Figure 3). When an antigen is presented together with MHC class II on the surface of APC and recognised by CD4+ T cells (Th0), the T cells differentiate towards Th1 or Th2 cells [3].

However, under certain circumstances exogenous peptides can be presented by MHC class I molecules and endogenous peptides can be presented by MHC class II molecules [13]. A few cell types have the capability to use the MHC class I restricted

presentation of exogenous antigens by a process called cross-presentation [13,14]. By this process, exogenous antigens such as soluble proteins, immune complexes and cellular antigens, are delivered into the MHC class I processing pathway and presented to CD8+ T cells. The major cell type known for its capacity to cross-present antigens is the DC, although other cell types including B cells and macrophages also have been reported to cross-present [14]. Thus, DCs can prime naïve CD8+ T cells (initiating immune responses for which immunological memory has not been established), regardless of antigen sources. Cross-presentation is involved in responses to viral infections, transplanted organs and tumor antigens [14].

For an effective T cell response, recognition at antigen (signal 1) by an MHC molecule is not sufficient; additional signals from co-stimulatory molecules (signal 2) are required [15]. The best characterized co-stimulatory molecules are two related forms of B7, B7-1 (CD80) and B7-2 (CD86) on APC. The receptors for B7 are CD28 and cytotoxic lymphocyte antigen-4 (CTLA-4) (CD125) expressed in the T cell membrane. CTLA-4 functions as a negative regulator of activation.

1.4 Tumor antigens

Due to genetic changes during the carcinogenesis, tumor cells will possess new antigens, which could be recognized by cells of the immunosurveillance network. Much of the information regarding the relationship between tumor cells and the immune system of cancer patients are achieved from in vivo and in vitro studies performed with melanoma [16]. A number of MHC class I restricted antigens have been identified on cancer cells recognized by CD8+ T cells, and more recently, it has been possible to identify MHC class II restricted tumor antigens recognized by CD4+ T cells [17,18]. TAs can be divided into different categories :

A) ***Tumor specific antigens (TSAs)*** are unique to tumor cells and normal tissue does not express these antigens. Typically, these antigens arise as a result of oncogenic transformation. TSA can be divided in *unique and shared TSA*. Unique TSA arise from point mutations of normal genes (such as β-catenin, caspase-8), and are generally expressed on the tumor where they were first identified. In humans, response to the unique TSA appears to be associated with a better prognosis [18]. A few numbers of Shared TSA have been identified, generated by mutations in k-ras or p-53, and are widespread in different cancers. Other TSAs include clonal rearrangements of Ig genes generating unique idiotypes in multiple myeloma and B-cell lymphomas.

B) ***Tumor associated antigens (TAAs)*** are not unique to tumor cells, but are expressed on somatic cells. TAAs represent the majority of tumor antigens. TAAs

can be further divided into; *Cancer testis (CT)* antigens such as MAGE, BAGE and NY-ESO-1, expressed in tumor cells but silent in normal (except germ) cells. *Tissue-specific Differentiation Antigens*, e.g., melanocyte-differentiation antigens including MART-1/Melan A, Gp100 and tyrosinase in melanoma [16]. These TAAs are shared between tumors and the normal tissue from which the tumor arose; most are found in melanomas and normal melanocytes. *Overexpressed/amplified self antigens* derived form non-mutated proteins and tumor suppressor genes are derived from non-mutated proteins and tumor suppressor genes (wild-type p-53, ganglioside proteins, epidermal growth factor receptor (EGFR, e.g., Her2/neu) and mucin-1). *Overexpressed oncofetal self antigens* are expressed on normal cells during fetal development, and down-regulated after birth. Reactivation of the embryonic genes that encodes these proteins in tumor cells results in their expression on the tumor cells. Examples include carcinoembroynic antigen (CEA) and epithelial cell-adhesion molecule (EpCAM). CEA belongs to the family of intercellular adhesion molecules [19], and is a surface-expressed TAA expressed at a high density on most malignant tumors of the gastrointestinal tract.

In addition, *tumor associated viral antigens* that contain T cell epitopes (Epstein-Barr virus, human papilloma virus, hepatitis B virus) are other examples of TAAs.

TAs recognised by cellular or humoral effector cells of the immune system are potential targets for antigen specific cancer immunotherapy.

Examples of TSA and TAA in colorectal carcinoma are given in Table 1.

Table 1. Tumor antigens in Colorectal Carcinoma [18,20].

Types of Tumor Antigens	**Tumor Antigens**
Tumor specific Antigens	
Mutated Antigen	p-53
	k-ras
	TGFβRII
Tumor Associated Antigens	
Cancer Testis Antigens	MAGE
Overexpressed self antigens	Normal p-53
	MUCIN-1
Overexpressed oncofetal self antigens	CEA
	EpCAM

1.5 Immune surveillance theory

The observation that the immune system could recognize and eliminate the host from cancer was initially proposed by Ehrlich [10] and introduced as the cancer immunosurveillance theory 1959 by Thomas [21] and then Burnet [22]. This theory suggests that the immune system recognizes and eliminate the host from cancer cells, which arise continously in the body. Initially, there was not much support for the immunosurveillance hypothesis, as no increase in incidence of either carcinogen-induced or spontaneous tumors, was observed in immunodeficient animal models or humans [23]. The initial studies were mainly performed in nude mice, with reduced number of T-cells and T-cells dependent immune responses. Additionally, the existence of αβ T cells and intact innate immunity, in the animal models was not established at the times, which lead to inconclusive results. However, recent studies have shown that disturbances in innate or adaptive immunity in mice render them highly susceptible to the development of chemically induced tumors and to the formation of spontaneous tumors of non-viral origin [24]. Although it is not possible to obtain direct experimental evidence for a cancer immunosurveillance process in humans, clinical data has supposed that this process also exists in human. Primary or secondary immunosuppressed individuals exhibit an increased risk for different cancers both of viral and non-viral origin [25,26]. Cancer patients have shown to develop spontaneous immune responses, involving both innate and adaptive immunity against the tumors that they carry [27,28]. In addition, a positive correlation has been made between the presence and location of T cells in a tumor and the prognosis of patients with a variety of different cancers [29-31]. Thus, the combined results now strongly supports the statement that immunosurveillance exists.

1.6 Innate and adaptive immune responses against tumors

NK cells

NK cells were first identified for their ability to kill tumor cells without the need for immunization or pre-activation [32]. Monoclonal antibody to the low-affinity Fc receptor CD16 (FcγRIII) are commonly used to identify them in the lymphocyte populations. Furthermore, NK cells are subdivided into two functionally different subsets: CD56-bright cells, being mainly cytokine producers and CD56-dim cells, being mainly killer cells [33]. Thus, in humans, NK cells are identified as CD16+CD56+CD3- cells. A distinguished feature of the NK cells is their ability to lyse either MHC mismatched cells (transplants) [34] or cells lacking or having low levels of MHC expression (virus-infected cells or cancer cells). The ability of NK cells to discriminate between normal and abnormal cells are attributed to the expression of inhibitory or activating cell-surface receptors [35].

Numerous animal studies have shown that NK cells play a critical role in the control of tumor growth and metastasis in vitro and in vivo [5,33,36]. NK cells can eliminate their targets by different mechanisms [5,33,36] (Figure 3):

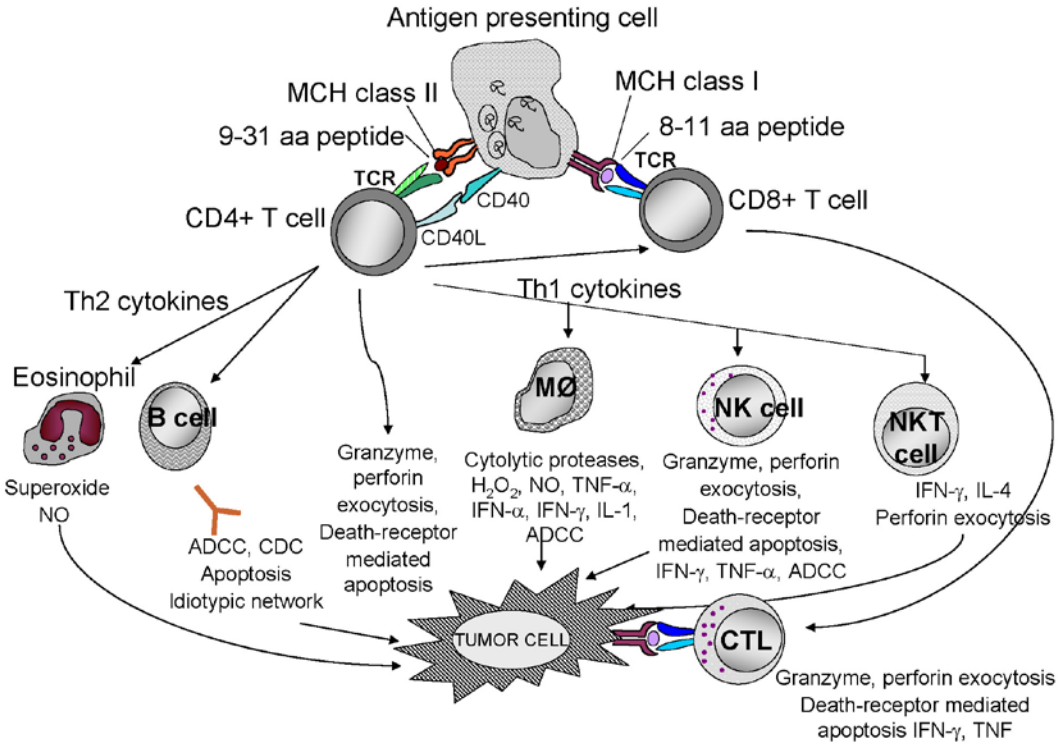

Figure 3. Schematic representation of potential effector mechanisms involved in the innate and adaptive immune responses against tumors. Immune responses against tumors comprise coordinated function of both innate and adaptive immunity. Tumor cells usually lack non-self epitopes and do not express costimulatory molecules. Tumor antigens (TAs) need to be presented by other types of cells in order to stimulate the immune system. Dendritic cells (DCs) are supposed to be the most potent type of antigen presenting cells (APC) that stimulate naïve T cells and play an important role in the induction of an immune response [6,7]. DCs can take up antigenic material from lysed tumor cells (not shown) [13], and deliver exogenous TAs peptides into MHC class II processing pathway to activate CD4+ T cells. CD4+Th1 cells producing IFNγ and IL-2 (Th1 cytokines) are required for maintaining CD8+ T cell function, and may also stimulate effector cells of the innate immunity. A subset of CD4+T cells may also be cytotoxic via direct or indirect pathways [11,12]. CD4+ Th2 cells secreting IL-4 and IL-5 (Th2 cytokines) provide help for B-cells producing antibodies, which may contribute to the tumor cell destruction by ADCC, CDC and induction of an idiotypic network response [37]. By a process known as cross-presentation, exogenous antigens are delivered also into the MHC class I processing pathway and presented to CD8+T cells by APCs [13,14]. Tumor-specific CD8+T cells (CTLs) are able to kill tumor cells by different mechanisms [38]. In addition, components of the innate immune system, such as NK cells [5,36] and macrophages may elicit direct cytotoxic functions against tumor cells by several mechanisms, see section 1.6. Furthermore, cytokines may exert antitumor activity by direct cytotoxic activity, or by the modulation of the immune response. (Modified illustration kindly provided by Szilvia Mosolits).

- *Granule- mediated exocytosis* is supposed to be the principal pathway used by NK cells to kill tumor (and virus-infected) cells. The cytotoxic granules of NK cells are complex organelles, harbouring perforin (a membranedisrupting protein), granzymes (serine proteases) and granulysin (cytolytic protein) which lyses target cells via apoptosis.

- *Death-receptor-mediated apoptosis.*

- *Cytokine production*. Activated NK cells produce a number of cytokines, including IFNγ, TFNα and GM-CSF. IFNγ have been prepared to mediate a direct cytotoxic activity, or by the modulation of the immune response. The above mentioned cytotoxic effctor mechanisms are also used by cytotoxic CD8+ T-lymphocytes (CTL) [38].

- *Antibody-dependent cellular cytotoxicity (ADCC)* (Figure 5).

- *"NK-DC cross talk"*. Emerging data has shown that NK cells and DC reciprocally activate one another during an immune response [39]. The relevance of NK cell activation by DC have been demonstrated in murine tumors models [40].

The *in vivo* role of NK cells antitumor activity in human is still unclear. However, several studies have found that high peripheral blood NK cell activity [36,41] or infiltration of NK cells in malignant tumors are associated with a favourable outcome [36,42]. Recent results implicating NK cells as important effectors in protecting human against tumors, comes from studies in patients with hematological malignancies, treated with hematological stem cell transplantation [34]. Allogenic NK cells derived from the donor were shown to be capable to mediate a graft-versus-leukemia effect in the recipient which protected against disease relapse.

Macrophages (CD14+) are a heterogenous population of cells derived from blood-borne monocytes that migrate into tissues where they undergo differentiation dependent on the microenvironment. Tumor-associated macrophages (TAMs) originate from circulating blood monocytes and represent a major component of the leukocyte infiltrates of many tumors and metastases [43].

Tumor regression by TAMs can be mediated by direct cellular cytotoxicity, by production of cytokines (IFNs, TNFα, IL-1, IL-6), reactive intermediates of oxygen (H_2O_2) or nitrogen (NO). Macrophages can also be effector cells in ADCC [9]. On the other hand, TAMs have been shown to play a role in tumor progression by releasing growth factors (EGF, TGFβ, platelet derived growth factor [PDGF]) or by effects on angiogenesis. In addition, TAMs can suppress T cell and NK cell mediated reaction, partly due to the production of IL-10, TGFβ and H_2O_2. Thus, it has been established

that TAMs have dual functions in their interaction with tumors - both in terms of regression and progression [44].

Natural killer T (NKT) cells are subset of mature T cells that co-express a TCR and NK cell markers such as CD161 [9]. In some cancer models, NKT cells have shown to contribute to anti-tumor immune responses via IFNγ-production [45].

γδT cells represent a minor population of peripheral T cells that have a TCR composed of γ and δ chains, where cell-specific antigens do not need to be processed by APCs and do not require presentation by classical MHC-molecules [9]. γδT cells can release cytokines, granulysin and lyse target cells, especially of hematological origin [46]. Additionally, γδT cells have recently demonstrated to function as an APC [47].

Cytotoxic T lymphocytes are CD8+ T cells expressing specific TCR that recognises TAAs on MHC class I molecules on the surface of tumor cells. CTLs represent the major effector cells of adaptive immunity and play a central role in antigen-specific antitumor immunity [38]. The activation of CTL occurs either via direct recognition of antigen on the tumor cell, or by cross presentation which subsequently prime CTL [13,14]. After activated, the CTL will undergo clonal expansion to antigen-specific CTL that will specifically lyse target cells that express the same peptide MHC class I complex. The CLT-mediated cytotoxicity is used in the same way as for NK cells.

CD4+ T cells are central to the development of immune responses against infections by activating antigen-specific effector cells, providing help for antibody production and recruiting cells of the innate immune system.

A subset of the antigen-specific CD4+ T cells may also be cytotoxic via direct or indirect pathways [11,12,48].

1.7 Tumor escape mechanisms with special focus on CRC

Despite the presence of innate and adaptive immune responses against tumor cells, the development of evident cancers occurs. Several different mechanisms by which tumor cells can escape immune recognition and elimination have been proposed [41], and some examples are given.

I. Alterations in tumor recognition

Complete loss or reduction of MHC Class I antigen expression is one strategy for tumor cells to avoid CD8 CTL recognition [49]. On the contrary, tumor cells that lose

MHC Class I expression are more susceptible to NK cell lysis according to the "missing self hypothesis" [50].

Downregulation of the MHC Class I molecules has been observed in several human tumors [51]. The most common mechanism for total loss of MHC Class I is alterations of β2-microglobulin expression, including CRC [20] (section 3.3).

II. Induction of T cell unresponsiveness

Several mechanisms have been described leading to alteration in T cell immunity in cancer patients. Many types of tumors have been reported to express functional Fas-ligand, which induces apoptosis of Fas-receptor expressing target cells, such as tumor infiltrating T cells in CRC [52]. Thus, peripheral tolerance induction may be mediated by *tumor-induced T cell death* [41]. T cell activation blockade can also be due to *receptor signaling defects*. Decreased expression of zeta chain of the TCR-associated molecule CD3 and the TCR-associated kinase p56lck has been found in a variety of human tumors including CRC [53]. Even though the TCR may be functional, T cell inhibition might still occur as a consequence of *lack of costimulation.*

The development of *peripheral tolerance in vivo* is one important mechanism by which tumor cells may evade immune recognition. Several mechanism can lead to induction of tolerance:

- T cell anergy induction may occur when T cells receive activatory signals in the absence of co-stimulation (signal 2), which can lead to peripheral tolerance. Many tumor cells lack the expression of important co-stimulatory molecules, such as B7 family members, that are necessary to achieve T-lymphocyte responses [54].
- Immune deviation, with a shift towards a Th2 response and inhibitory cytokine profile (IL-4, IL-10), has shown to correlate with disease progression in CRC [55].
- CD4+CD25+regulatory T cells (Treg) is a subset of T cells which co-express CD4 and CD25 (αchain of IL-2 receptor), are suggested to play an important role in induction of tolerance [56]. Recent studies have shown that cancer patients have increased numbers of circulating and TAA CD4+CD25+Treg cells [57], including CRC patients [58]. In addition, human Treg cells have shown to be functional , as shown by inhibition of NK cell-mediated cytotoxicity in vitro [58] and produces immune suppresive factors such as IL-10 and TGFβ.

III. Immunosuppressive factors

Colon carcinoma cells and tumor associated macrophages can produce IL-10 and TGFβ, cytokines that suppress cellmediated responses [44,59]. High concentrations of TGFβ are associated with poor response to immunotherapy in CRC patients [60].

Cancer cells can produce vascular endothelial growth factor (VEGF) which suppress DC differentiation and maturation [61]. VEGF is a key regulator of normal and pathological blood vessel growth [62]. Thus, in addition to the immune suppressive effects on the tumor micro environment, VEGF contributes to angiogenesis and vascularization of the tumor.

2 IMMUNOTHERAPY AGAINST CANCER

Over the past decade, immunotherapy has become increasingly important in the management of malignant disease. The technological advances in the last few years have led to a great increase in the number of and characterization of TAAs that are currently available for clinical applications. Current immunotherapeutic strategies can be divided in non-specific and specific modalities.

2.1 Non-specific immunotherapy

In non-specific immunotherapy, immunomodulating agents are administered with the aim to obtain a generalised immune response. The mechanisms of action of these substances are not completely understood. Non-specific immune responses are produced by cells of the innate immune system such as NK-cells, neutrophils and monocytes/macrophages.

2.1.1 Bacille Calmette Guerin (BCG)

The intravesical instillation of BCG in patients with superficial bladder carcinoma has been established since decades and generates an inflammatory process, which attracts APCs. In terms of colorectal cancer, BCG has been tested in laboratory and clinical trials, usually with chemotherapy without any clinical benefit [63]. BCG has also been used as an adjuvans in tumor vaccination.

2.1.2 Recombinant cytokines

The systemic administration of recombinant cytokines (e.g. IL-2, IFNα and IFNγ) has been used widely in humans for treatment of different malignancies.

Interferon-alpha (IFNα)

Interferons (IFN) display antineoplastic efficacy in vitro and in vivo by direct and indirect modulation of the host immune cell functions.

IFNα production can be induced by a number of agents (viruses, cytokines, etc.) and is mainly produced by leukocytes. Several studies have shown that IFNα may increase the expression of cell surface antigens including TAAs, MHC class I and II molecules and Fc-receptors on effector cells [8,64]. In addition, IFNα can exert enhanced ADCC and increased functions of NK cells [65] and antiangiogenetic activity [64].

IFNα is used in the treatment of chronic myelogenous leukemia, malignant melanoma and alone or in combination with interleukin-2 (IL-2) in treatment for renal cell carcinoma [66]. *Ex vivo* IL-2 activated peripheral lymphocytes (also termed as lymphokine-activated killer (LAK) cells together with high dose IL-2 produced modest anti-tumor activity in patients with renal cell cancer and melanoma [67], but with significant toxicity. Treatment of CRC with IL-2 and IFNα has proved ineffective [20,68].

Early *in vitro* data suggested synergism between IFNα and 5-fluororuracil (5-FU) for killing of colorectal cancer cell lines [69]. Combination of IFNα and 5-FU in CRC patients induced a response rate of 26–42%. However, these phase II results could not be confirmed in randomized trials [69].

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

The glycoprotein GM-CSF was the first CSF to be purified cloned and expressed using recombinant DNA technology [70]. Endogenous GM-CSF is produced by monocytes/macrophages, fibroblasts, endothelial cells and T-lymphocytes [8]. The biological effects of GM-CSF are mediated via binding to receptors consisting of a GM-CSF specific low-affinity α chain, and a signalling β chain that is shared with the receptors for IL-3 and IL-5 [71].

GM-CSF has pleiotropic effects and regulates the proliferation and differentiation of haematopoietic progenitor cells and modulates the function of mature haematopoietic cells [70]. GM-CSF induces the recruitment, development and differentiation of DCs [72] as well as activation of macrophages leading to markedly enhanced antigen presentation. By augmenting antigen presentation to lymphocytes by DCs, GM-CSF stimulates T-cell immune response, which provides the basis for its potential role as a vaccine adjuvant, including for CRC [73,74]. The capacity of GM-CSF to augment an antitumor immune response is most likely due to the ability to enhance antigen presentation by DCs *in vivo* [75]. In addition, GM-CSF also augments the cytotoxic capacity of neutrophils and monocytes, and increases NK cell functions directly or by stimulating monocytes to enhanced production of γ-IFN, IL-12 and IL-15 [73]. Thus, there are considerable effects of GM-CSF on the immune response that might be utilized not only as a vaccine adjuvant, but also together with monoclonal antibodies for the treatment of malignant tumors [73,76] (II, III).

In contrast to traditional drugs, which effects are based on the identification of the maximum tolerated dose (MTD), there might be a complex dose-response relationship between the various functions and the dose of recombinant proteins that are administered. For some cytokines, a bell shaped relationship between the dose and biological effect has been described instead for a linear [77]. Regarding GM-CSF, there seems to be a linear relationship between proliferation and the expansion of cells and the dose of GM-CSF, whereas such a correlation might not exist for

functional activation, such as enhancement of cytotoxic activity [78,79]. Thus, it is of importance to establish the optimal therapeutic dose (OTD), which may not be the same as MTD. Indeed, GM-CSF seems to have dual effects, as it might also induce immune suppression [73,80].

Therapy with cytokines, such as α-IFN and GM-CSF can induce development of antibodies which not only bind but also neutralise the biological activity and alters the pharmacokinetics of the administered cytokine [81-83].

2.2 Antigen-specific immunotherapy

Antigen-specific immunotherapy aims to generate an immune response to a specific antigen. The functioning of the antigen-specific immunotherapy is based on activation of T-cells and B-cells. Treatment with monoclonal antibodies and vaccination belongs to this immunotherapeutic approach.

2.2.1 Monoclonal antibodies

Monoclonal antibodies (MAbs) are produced by a single clone of B-cells, and are monospecific and homogenous. The mouse hybridoma technology described in 1975 by Köhler and Milstein, was the first milestone in antibody engineering [84]. MAbs are produced by fusion of spleen cells from an immunized animal with myeloma cells, to obtain hybridoma-cells, and then further screened for the production of antibodies against the specific antigen and propagated in laboratory cultures or in mice [85]. The majority of MAbs produced by hybridoma-technique are murine antibodies (mMAb). One disadvantage with murine MAbs is their immunogenicity, leading to the induction of human anti-mouse antibodies (HAMA), which accelerate the clearance of the MAb from the blood, and might cause allergic reactions [76,86-88]. Another major drawback of murine MAbs is that the Fc component of the mouse antibody molecule (the biologically active component), interacts less efficiently with human effector functions as compared to their human counterpart [89]. It is thought that the most immunogenic regions of antibodies are the C domains [86]. Genetic engineering technology has made it possible to decrease the immunogenicity of MAbs by development of; chimeric [90]; humanized/complementarity determining region (CDR) grafted [91] and fully human antibodies (Figure 4).

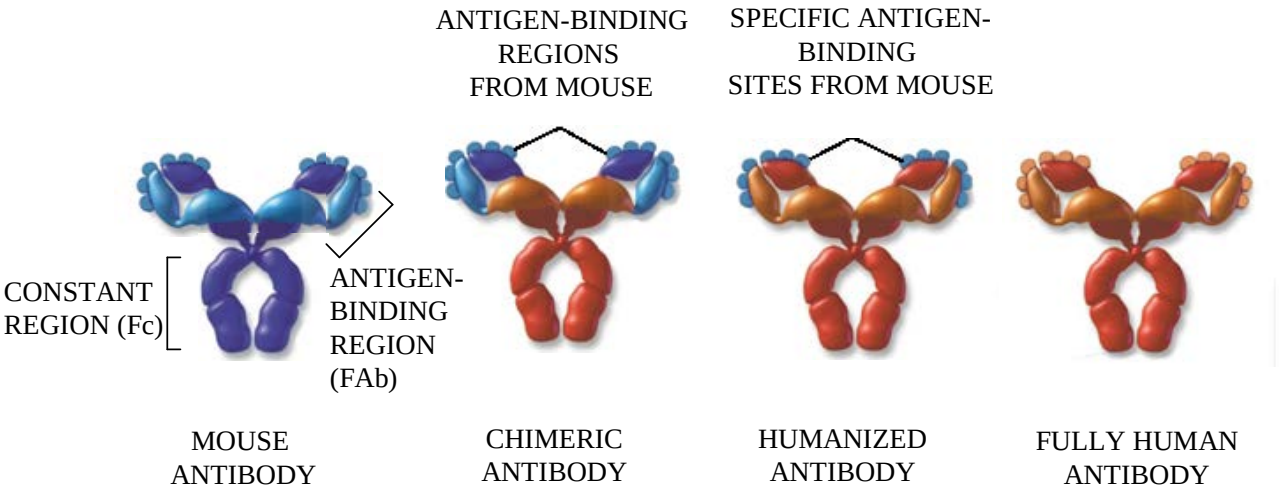

Figure 4. Types of monoclonal antibodies. Chimeric antibodies are 60%–70% human and consist of the murine variable regions (antigen recognition), fused to the constant or effector part of a human antibody. Humanized antibodies are about 90%–95% human, and are made by grafting the hypervariable region, or CDRs, of the murine (determines antibody specificity), onto a human antibody backbone. Fully human antibodies are produced by phage display libraries or transgenic animals. (Reprinted with permission from Foster Medical Communications)

Examples of chimeric monoclonal antibodies (cMAbs) include rituximab (anti-CD20 receptor MAb) [92] and cetuximab (anti-EGFR MAb) [93].

The anti CD52 MAb alemtuzumab (Campath-1H) was the first humanized monoclonal antibody (huMAb) produced [94] and other examples are trastuzumab (anti-Her-2/neu MAb) [95] and bevacizumab (anti-VEGF MAb) [96]. Phage display technology allows the presentation of large peptides and proteins (including antibodies) on the surface of a bacteriophage (viruses that infect bacteria) [97], and is currently a widely used technique for antibody display and library screening [85,98]. The production of fully human antibodies by phage display antibody libraries or transgenic animals has enabled the selection and identification of fully human MAbs, as well as the improvement of MAb affinity [85,98]. There are also increasing number and variety of recombinant immunoglobulin-based structures, such as recombinant antibody fragments, anti-body like structures and bispecific antibodies [85,89] in order to evaluate their potential for improving the efficacy of whole antibodies. Small antibody fragments might improve antibody penetration into poorly vascularized tumor areas, although their small size can lead to poor retention of the tumor and rapid clearance. Bivalent antibody fragments are studied as a method for increasing binding affinity and tumor retention [85].

Bispecific MAbs are produced for targeting the recruitment of effector cells to a tumor. One arm of the antibody binds the TAA and the other binds to effector cells. MDX-210 is a bispecific antibody fragment that targets Her-2/neu and CD64 (monocytes, activated granulocytes). Clinical trials have showed that MDX-210 has activity in patients with solid tumors, such as breast, ovarian, and prostate cancer [99].

Immunogenecity of MAbs

Most of the murine MAbs lead to the formation of a human anti-mouse antibody (HAMA) response [86,87,100]. This HAMA response may impair the pharmacokinetic profile of the antibody, leading to rapid clearance of the MAb, and preventing repeat administrations due to allergic reactions. However, MAb therapy can also induce an idiotypic network response, that can be beneficial [37] (section 2.2.2). The production of chimeric MAb, with human constant regions, humanized MAb, retaining only murine CDRs, and fully human MAb made from phage libraries or transgenic mouse have reduced the murine content and the immunogenicity of the MAbs. In general, 40–80% of patients with solid tumors develop a HAMA response, whereas the incidence is lower in patients with B cell malignancies [100].

In our trials, repeated infusions of the murine anti-EpCAM MAb induced gradually increasing titers of HAMA and was detected in 95%–100% of the patients [101,102]. However, no significant impairment of the pharmacokinetic variables were noticed and more important, the incidence of allergic reactions were low as long as the MAb was given alone. [102]. The incidence of human anti-chimeric antibody (HACA) responses is reduced to approximately 40% in patients with solid tumors treated with chimeric MAbs [86,87]. HACA formation against chimeric anti-EpCAM MAb, used for therapy in this thesis (III), has been reported in 10% of patients [103] and only recognizing the variable regions of the chimeric molecule. Low HACA responses are reported for cetuximab (5%) [87]. Humanization of variable regions appears to decrease immunogenicity further as no human anti-humanized antibody (HAHA) responses have been reported for bevacizumab, humanized anti-EGFR Ab EMD7200 (matuzumab) or fully human anti-EGFR MAb ABX-EGF (panitumumab) [87,104,105] up to date. In addition, genetically engineered MAbs have longer biological half-lifes ($t_{\beta½}$ = elimination half-life), see examples in Table 2.

Table 2. Elimination half-life ($t_{\beta½}$) following a single dose of different types of monoclonal antibodies [9,102-105]

Antibody	**$t_{\beta½}$**	**Single dose**
murine anti-EpCAM	~20 hrs	400 mg
chimeric anti-EpCAM	~100 hrs	10–40 mg
chimeric cetuximab	~66–97 hrs	250–400 mg/m^2
humanized EMD 7200	~116 hrs	400-800 mg
humanized bevacizumab	13–21 days	3 mg/kg
fully human ABX-EGF	6 days	2.5 mg/kg
Human IgG	21 days	(range 15–30 days)

2.2.2 Mechanism of action of unconjugated MAbs

The mechanism of action of any particular unconjugated MAb depends on characteristics of the MAb as well as characteristics of the targeted antigen. Furthermore, as discussed below, the interaction between the therapeutic MAb and its target antigen may involve both immune and non-immune-mediated mechanisms, and the current knowledge on the mechanisms of action of unconjugated MAbs, is based mainly on in vitro studies: the relative contribution of each of them in vivo, when treating patients, is still unclear.

The isotype and subclass of the MAb is of importance for mediating tumor cell death. Immunoglobulin IgG1 is most effective in recruiting effector cells, such as NK-cells and monocytes/macrophages, as well as activating complement [106,107]. IgG3 is also active in ADCC and stronger than IgG1 in activating CDC, however, complement activating of IgG3 may be too strong leading to unwanted adverse effects. The Fc-part of murine MAbs interacts less efficiently with human effector functions as compared to the human counterpart, favoring the use of chimeric or humanized antibodies [89,106]. However, if the antibody are required to activate or block a receptor, then human IgG2 or IgG4 would probably be more appropriate [89].

The impact of the antigen-binding affinity of IgG molecules on their tumor-localization ability is a matter of debate [106,108,109]. If the tumor-antigen is expressed on micrometastases or circulating tumor cells such as leukemic cells, a high affinity MAb (i.e., within the range of 10^{-9} to 10^{-11} M) might be a benefit. In contrast, when a MAb is going to penetrate into a bulky tumor mass, a low to intermediate affinity MAb (i.e., within the range of 10^{-7} to 10^{-9} M) might be an advantage [110]. Use of high-affinity MAbs in patients with solid tumors. has showed both unacceptable [111] and acceptable adverse effects [109].

Characteristics of the antigen that contribute to the mechanism of action of the MAb include its function, its cell-surface density and tissue distribution. Preferentially, the antigen should be both stably and homogenously expressed by tumor cells, is expressed at very low levels in normal tissues, and with the antigen expression preserved in metastatic deposits. Ideal, it might be expressed on the cell surface and should not be modulated/secreted, or be processed and presented by MHC proteins [63,107]. Furthermore, structures that spontaneously induce a tumor immune response (humoral or cellular) and/or are involved in growth processes of the tumors should be considered as antigen [107].

Immune-mediated mechanisms

I. Antibody-dependent cellular cytotoxicity (ADCC)

ADCC is thought to be a dominant contributor to the anti-tumor activity of unconjugated MAbs, and the importance of this effector mechanism is strongly supported for certain MAbs by animals models [112] and genetic evidence [113]. ADCC is triggered when the Fc region of the target-bound antibody (especially the IgG1 and IgG3 subclasses) is recognized by Fcγ receptor bearing effector cells (NK-cells, monocytes/macrophages and granulocytes) [106,107,114] (Figure 5).

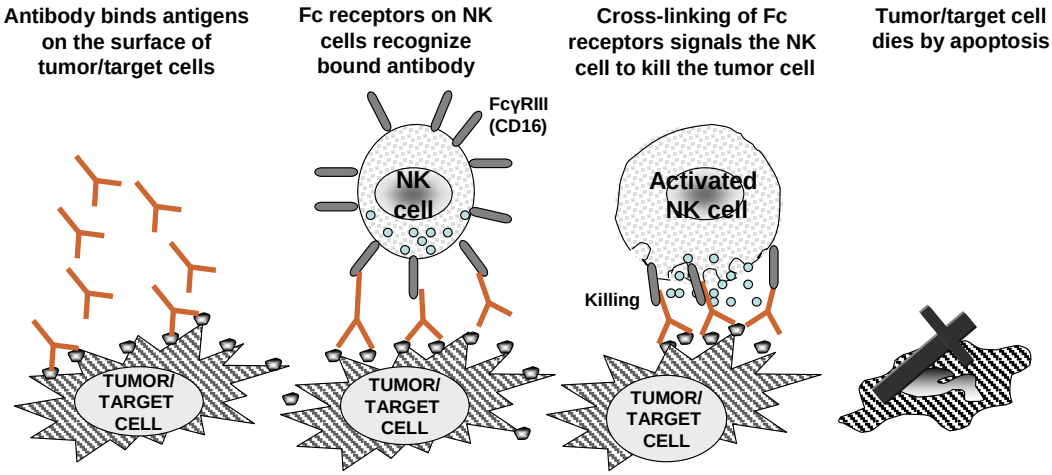

Figure 5. Antibody coated tumor/target cells can be killed by FcγR-bearing cells, such as natural killer (NK) cells, in antibody-dependent cell-mediated cytotoxicity (ADCC) [114]. NK cells express the activation receptor FcγRIII (CD16) on their surface, which recognize the Fc domains of IgG1 and IgG3 subclasses. When NK cells encounter cells coated with IgG antibody, they become activated and kill rapidly the tumor/target cells by the release of cytoplasmic granules containing perforin and granzymes. FcγRIIb is an inhibitory receptor expressed on macrophages (but not NK cells) that co-ligates to

FcγRIII activation receptors, leading to inhibition of FcγRIII signaling (not shown) [9,112], thus inhibiting effector cell activation.

The low affinity FcγRIIIa (CD16) is suggested to be the most important Fc binding receptor inducing ADCC, with the participation of the high-affinity monomeric FcγRI (CD64) and FcγRIIa (CD32). However, binding to the FcγRIIb has been shown to be an inhibitory receptor expressed on macrophages [112]. NK cells are thought to be the main effector of ADCC, although monocytes bearing the FcγRI (CD64) receptor may also contribute [107,115]. The first evidence that the Fc-Fcγ receptor interaction is important for the antitumor activity of an antibody *in vivo* came with the development of mice that lack FcγRI and FcγRIII [116]. The importance of the Fc-Fcγ receptor interaction for antitumor activity has subsequently been shown for clinical antibodies such as huIgG1 trastuzumab and cIgG1 rituximab in animal models. The antitumor activity of both trastuzumab and rituximab was greatly reduced in mice that lack the activation receptors (FcγRI and FcγRIII) [112]. ADCC is supposed to be the main effector function of the mouse anti-EpCAM antibody and its chimeric variant [103,114,117,118], used for therapy in this thesis.

II. Complement-dependent cytotoxicity (CDC)

Complement-dependent cytotoxicity (CDC) of tumor cells is a strong effector function, but is less well documented than ADCC. Yet, chimeric and humanized MAbs of subclass IgG1 has shown to activate the complement cascade in vitro [2]. However, tumor cells might be protected from CDC by the surface expression of complement-regulatory proteins (CRP) CD46, CD55 and CD59 that inhibit the complement cascade, and reduce the effect of the MAb [2]. A series of studies both in vitro and in vivo suggest that CDC is of importance for the mechanism of action of rituximab [119], and the complement resistance may be overcome by anti-CRPs antibodies [120].

Anti–EpCAM mMAb antibody (see section 3.6.3) activate the complement system in vivo [121]. In our own material, we have been able to demonstrate deposition of complement at the site of the antigen in biopsies from metastases, after treatment with anti-EpCAM mMAb [122].

III. Induction of an immune network response

In accordance with the idiotypic network hypothesis [37], postulated by Niels Jerne 1974, an epitope of a particular antigen may induce the synthesis of an antibody (idiotypic antibody=Ab_1) specific for that antigen.

The antigen binding part (V region) of a therapeutic MAb (Ab_1), can act like an antigen itself, and elicit an immune response that leads to the production of anti-idiotypic antibodies (Ab_2). These anti-idiotypic antibodies (Ab_2) are directed against

epitopes on the heavy chain component of the variable region, on the light chain component, or on a surface made up of parts of both chains [123] of Ab_1. Amino acid sequences within the CDR regions of a subset of Ab_2s have homology with the therapeutic antibody (Ab_1), thus represents an internal image of the three-dimensional structure of the native antigen (Figure 6).

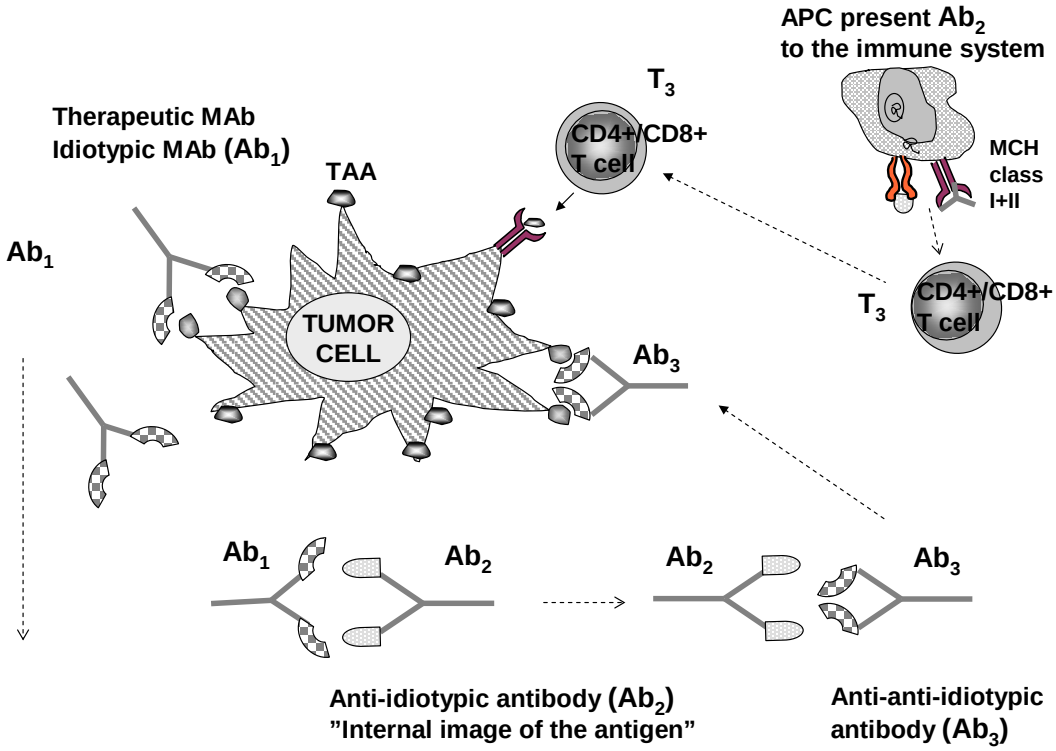

Figure 6. Hypothetical schematic presentation of an idiotypic network response [37]. After presentation to the immune system, the therapeutic MAb=idiotypic MAb (Ab_1) may elicit an anti-idiotypic humoral (Ab_2) response against the idiotype of Ab_1. A subset of Ab_2 represents a positive imprint or "internal image" of the three dimensional structure of the nominal antigen. Ab_2 may subsequently induce an anti-anti-idiotypic immune response (Ab_3). Some of the Ab_3 population may be functionally identical to Ab_1, i.e. recognizes the original antigen, and may bind to the TAA and destroy the tumor cells by ADCC, CDC and/or apoptosis. T cells (T_3) recognizing Ab_2 and the nominal antigen might also be induced by MAb treatment [124].

Ab_2 in turn can stimulate the production of anti-anti-idiotypic antibodies (Ab_3) that may be functionally identical to Ab_1, thus be able to bind directly to the tumor antigens and mediate cytotoxicity via ADCC or CDC.

The network response theory also postulates the induction of an anti-idiotypic and anti-anti-idiotypic cellular response of T cells (T_2 and T_3, respectively). Thus, the therapeutic Ab_1 may induce both a humoral and a cellular anti-tumor immune response, that seems to be of clinical benefit [86,100,101,124-126].

Nonimmune-mediated effector functions

In addition to the immune-mediated effector functions, therapeutic MAbs can kill tumor cells directly by diverse array mechanisms. Antibodies directed targeting growth factor receptors achieve their anti-tumoral effects by ***binding to the receptor*** and act as ***antagonistic ligands***. The epidermal growth factor receptor (EGFR) belongs to the erbB family of four closely related cell membrane receptors, and both EGFR (erbB-1, HER1) and HER2 (erbB-2) are targets for cancer therapeutic MAbs. Cetuximab (Erbitux®) is a chimeric IgG1 MAb that binds competitively to the extracellular domain of EGFR, with a higher affinity than endogenous EGF, thus ***blocking*** ligand-induced phosphorylation of EGFR, and results in the inhibition of cell cycle progression, ***stimulation of apoptosis***, inhibition of angiogenesis and prevention of metastastis formation [127]. While immune mechanisms did not seem to play an important role in vitro, it is possible that they may contribute to its antitumor activity in vivo [127]. Trastuzumab (Herceptin®) is a humanized IgG1 MAb that binds to the extracellular domain of HER2, and in experimental system trastuzumab ***inhibits tumor cell proliferation***, angiogenesis by reduced production of VEGF and the growth of HER2-expressing tumors [128]. Other examples of MAbs targeting HER2 are MDX-H210, a bispecific antibody that target HER2 and CD64 [99].

Unconjugated MAbs can also induce antitumor activity by ***blocking structures or signaling substances*** involved in the growth of tumors or metastasis formation. Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factor (VEGF) that binds to VEGF receptors on vascular endothelial cells. Antibodies directed at VEGF bind specific epitopes, ***blocking the interaction*** of VEGF with its receptors, and thus preventing VEGF signaling through its receptors. Bevacizumab (Avastin®) is a humanized MAb that neutralize VEGF [62,96].

Unconjugated MAbs as single agent or combination therapy

The majority of the approved unconjugated MAbs, has shown to be effective when used as single agent therapy [129,130]. Rituximab (anti-CD20) and trastuzumab (anti-HER-2/neu) were the first MAbs to demonstrate single agent activity in CD20+ B cell lymphomas or HER-2/neu+ breast cancer, respectively [131,132]. In addition, unconjugated MAbs may also potentiate the effect of chemotherapy [129,133-135]. Furthermore, maintenance therapy with unconjugated MAbs following adjuvant chemotherapy has shown to improve the disease free survival in breast cancer patients [136] and is under investigation both in solid and hematological malignancies.

To enhance the immunological effector functions, unconjugated MAbs have been combined with cytokines in hemtological malignancies and CRC [76,137] (II, III), and studies are currently underway to investigate trails combining this approach.

Furthermore, MAbs targeting growth factor receptors, has shown in experimental and clinical models, to enhance the effects of radiation [138].

2.2.3 Conjugated MAbs

MAbs might be conjugated to chemotherapeutics, enzymes, toxins or radioisotopes. The rationale behind conjugated MAb therapy is to achieve a delivery of these agents to tumor sites, while sparing normal tissue. In addition, unconjugated MAbs with proven antitumoral effect by it self, have been combined with radionuclides in an attempt to further improve their therapeutic efficacy [139].

A number of standard ***chemotherapeutic agents*** have been conjugated to MAbs (e.g., doxorubicin and methotrexate) [140]. Gemtuzumab ozogamicin is a combination of cytotoxic agent (calicheamicin) and anti-CD33 MAb (Mylotarg®) which are used for patients with acute myeloid leukaemia [141].

Antibody directed enzyme prodrug therapy (ADEPT) employs the administration of a non-cytotoxic prodrug that is activated in a tumor by an enzyme conjugated to a tumor-specific MAb [63]. This approach has been undergone phase I evaluation for patients with CRC, by using A5B7 anti-CEA antibody conjugated to carboxypeptidase G2 (CPG2) and the prodrug bis-iodo phenol mustard [142].

Radioimmunotherapy (RIT) using MAbs labelled with radionuclides that is delivered to tumor sites. High-energy radionuclides that emit β particles, such as yttrium-90 (^{90}Y) and iodine-131 (^{131}I), are the most widely used radionuclides for RIT [143] . The preference for using β-emitters in RIT is due to their millimeter path length that enable the killing of antigen-negative tumor cells (so-called bystander phenomenon or crossfire) [143]. RIT has mainly been used for the treatment of lymphoid malignancies. Radiolabelled anti-CD 20 MAbs (^{90}Y-ibritumomab tiuxetan (Zevalin®) and ^{131}I-tositumomab (Bexxar®)) have shown efficacy for the treatment of non-Hodgkin´s lymphoma (NHL), including rituximab-refractory patients [139].

Solid tumors are targeted less effective than hematological malignancies due to limited vascular supply, raised interstitial pressure, and a heterogeneous uptake of the antibody in the tumor [144]. Furthermore, they have lower radio sensitivity. The therapeutic efficacy of RIT in CRC has been evaluated in some phase I/II RIT trials, mostly in patients with advanced disease [145]. In the majority of these studies, ^{131}I-radiolabeled anti-CEA antibody preparations have been used [145]. Single administration of a ^{131}I-labeled humanized anti-CEA antibody (hMN-14) in CRC patients with either small-volume disease or in a semi-adjuvant setting after surgical resection of liver metastases, showed promising results [146]. Since tumor uptake increases with decreasing tumor size [145], RIT might have an effect in the adjuvant setting of CRC patients.

2.3 Active specific immunotherapy

Active specific immunotherapy principally involves the use of cancer vaccines with the aim to evoke a tumor-specific immune response in cancer patients. When using vaccination as treatment for cancer the tumor cells have to express tumor antigen on their surfaces, and to enable the immune system to distinguish and kill the tumor cells, they need to be different from normal cells. There are many questions to address in finding effective cancer vaccine strategies, i.e. type of vaccine, schedule and the use of adjuvants.

Among several different ways to create a tumor vaccine, clinical vaccine trails in CRC have used; ***whole tumor cells*** (modified in vivo or ex vivo), ***TAAs*** such as CEA or EpCAM, or ***anti-idiotypic antibodies*** [147]. In the adjuvant setting, stage II-III CRC patients have been vaccinated with autologous tumor cells mixed with BCG as adjuvant (OncoVAX) [148,149]. Subgroup analysis form these studies suggests that patients with stage II colon cancer might benefit from vaccination, with improvement in disease-free survival [149] or overall survival in immune responders [148]. Using Newcastle-disease virus (NDV) as an adjuvant has showed to significantly improve overall survival in patients whit stage I-IV CRC [150].

Recombinant CEA protein has been used for immunization of stage I-III CRC [151]. A significant augmented humoral and cellular immune response was observed in patients treated with the addition of GM-CSF compared to patients without GM-CSF. Furthermore, a positive correlation between the anti-CEA IgG titer and overall survival was suggested [151]. In addition, no clinical manifestation of autoimmune reactions or other long-term adverse effects was observed.

Another vaccine approach is insertion of a ***tumor antigen-derived DNA sequence into attenuated virus*** (e.g., canary pox) unable to replicate in mammalian cells [152]. ALVAC-KSA is a recombinant canarypox virus expressing the full-length EpCAM gene [153]. Vaccination with EpCAM expressed in ALVAC vector induced a strong anti-EpCAM-specific Th1-type response when administered together with GM-CSF, in stage I-III CRC patients [154].

An adjuvant is a substance which enhance the immune response and addition of adjuvants are often crucial to induce a sufficient immune response in vaccination. There are various types of adjuvants used in immunotherapy, ranging from microbial, chemical, and cellular components to proteins and cytokines [155]. Examples include incomplete Freund´s adjuvant (IFA), BCG, aluminium-based salts (alum) and NDV. Cytokines, such as GM-CSF, have been demonstrated to augment both humoral and cellular immunity by several mechanisms [73]. Preferable, stimulation towards a Th1 type response rather than a Th2 type is desirable for antitumor activity [74].

3 COLORECTAL CANCER

3.1 Epidemiology and carcinogenesis

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer deaths worldwide. The WHO estimates that nearly one million new cases occur early, with half a million deaths [156,157]. Although CRC is the second most common cause for cancer related death in developed countries, recent studies show that one-third of new CRC cases occur outside industrialized countries [158]. In Sweden, the annual incidence of CRC is approximately 62 cases per 100 000 inhabitants (64/100 000 males and 60/100 000 females) which makes it the second most common cancer in both sexes, after breast cancer and prostate cancer, respectively, with approximately 5 550 new cases/year [159]. Median age at CRC-diagnose in Sweden is 70–75 years. The 5-year relative survival rate in Sweden irrespective of the stage and gender, has improved significantly for colon carcinoma from 39.6% in 1960–1964 to 57.2% in 1995–1999 and for rectal cancer from 36.1% to 57.6%, respectively [160] (Figure 7).

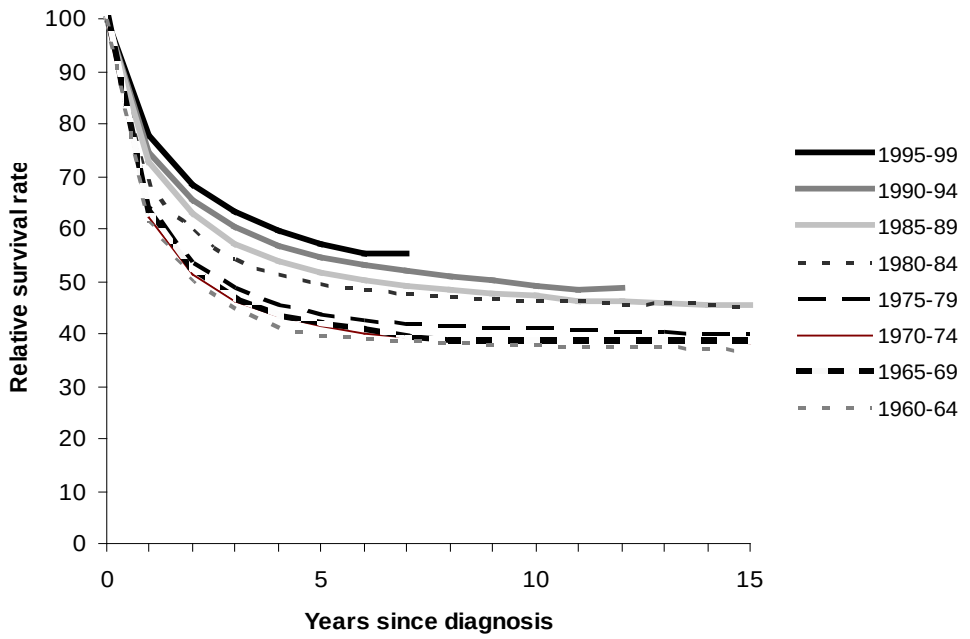

Figure 7. Cumulative relative survival rate of coloncancer in Sweden for the year 1–15 after diagnosis, comparison between 5-year time periods from 1960 to 1999. (Modified from [160])

Cancer cells acquire six essential alterations in cell physiology that dictate malignant growth; [1]Self-sufficiency in growth signals, [2]Insensitivity to growth-inhibitory signals, [3]Evasion of programmed cell death (apoptosis), [4]Limitless replicative potential, [5]Sustained angiogenesis, and [6]Tissue invasion and metastasis [161]. The model for development of CRC represents a classical example of multistep carcinogenesis including a number of genetic and epigenetic alterations from a normal epithelium through the sequential development of early, intermediate, late adenoma and adenocarcinoma [162].

In ~ 85% of CRC, the genetic instability is seen at the chromosomal level, as a consequence from a series of genetic changes that involve the activation of oncogens such as Kirsten-ras (K-ras), and inactivation of tumor suppressor genes, such as adenomatous polyposis coli gene (APC) and p53 (CIN pathway). CIN tumors are characterized by aneuploidy, allelic imbalance (most commonly involving chromosomal arms 5q, 8p, 17p and 18q), amplifications and translocations [163]. The remaining 15% of CRC are characterized by microsatellite instability (MSI), reflecting inactivation of the mismatch repair (MMR) genes [164].

Between 5% and 15% of all CRC is estimated to be due to heredity, and in this case, the onset of cancer occurs at earlier age than the sporadic forms. Familial adenomatous polyposis (FAP) and hereditary non-polyposis (HNPCC) are the two major inherited CRC syndromes.

3.2 Staging

To select the optimal therapeutic strategy for a patient with CRC, a correct clinical and pathological staging is of great importance. Rectal cancer was among the first malignant diseases in which an attempt was made to estimate prognosis following surgery by grading the extent of spread of the tumor according to the pathological staging system first developed by Duke in 1932 which classified rectal tumors from A to C. Seventeen years later the staging system was enlarged to include both rectal and colon cancers [165]. This staging system is based on the depth of extension of the carcinoma through the bowel wall and the presence or absence of lymph node metastases (Table 3). Several modifications has been developed. The TNM staging system [166] of the AJCC and UICC is now recommended as standard for CRC staging [167] and for prediction of five year survival (Table 3). In our studies we used modified Astler-Coller classification [168] in Papers I–III and AJCC/UICC staging in Paper VI.

Table 3. AJCC/UICC stage groupings for colorectal cancer and comparison of other staging and classification systems.

AJCC/UICC stage	**TNM**	**Modified Astler-Coller** [168]	**Dukes** [165]	**5-year overall survival** [166,169,170]
I	T1-2, N0, M0	A-B1	A	80–95%
IIA	T3, N0, M0	B2	B	72–75%
IIB	T4, N0, M0	B3	B	65–66%
IIIA	T1-2, N1, M0	C1	C	55–60%
IIIB	T3-4, N1, M0	C2-3	C	35–42%
IIIC	Any T, N2, M0	C1-3	C	25–27%
IV	Any T, Any N, M1	D	D	0–7%

T (primary tumor)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
TIS	Carcioma in situ (intraepithelial cr invasion of lamina propria)
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the subserosa or into the nonperitonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum

N (nodal status)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in 1 to 3 regional lymph nodes
N2	Metastases in 4 or more regional lymph nodes

M (distant metastases)	
MX	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases

3.3 Prognostic factors in CRC

The 5-year survival for patients with CRC is directly related to *the clinical and pathological staging* at the time of diagnosis (Table 3). It varies between 80%–95% for stage I, 65%–75% for stage II, 25%–60% for stage III and less than 7% for stage IV [170].

As a general consensus it has been suggested that a minimum of 12 lymph nodes need to be examined for adequate staging [167]. Additionally, when controlled for the number of lymph nodes with metastases, survival has shown to increase with the total

number of examined lymph nodes, also within the node-negative group of patients [171].

Examples of other proven prognostic factors than pathological stage for CRC patients with complete resection of the primary tumor and for patients with unresectable primary tumor or metastatic disease are given in Tables 4a and 4b, respectively.

Table 4a. Prognostic factors in CRC patients with complete resection of primary tumor (unfavourable level of covariates is shown in paranthesis) [167,170,172,173].

Factor	
• *Disease stage* according to AJCC/UICC stage (see Table 3)	• *Perforation* of the bowel wall by tumor (present)
• *Histological grade* (high)	• *Extension* of surgery (residual tumor [macroscopic or microscopic] present after surgery, especially in rectal cancer)
• *Venous vessel* invasion (present)	• *Preoperatively CEA* levels (elevated)
• *Lymphatic vessel* invasion (present)	• *Postoperatively CEA* levels (elevated)
• *Comorbid disease* (present)	

Failure of the CEA to decrease to normal levels postoperatively following a complete resection suggests residual tumor or the presence of occult metastatic disease [173].

Additionally, CA 19–9 is the second most common serum tumor marker used in CRC and high preoperatively levels have shown to be associated with adverse outcome [174]. The combination of both preoperatively elevated CEA and CA 19–9 have been shown to identify patients in stage II CRC with poorer prognosis than those with normal levels of these markers [175].

Table 4b. Prognostic factors in CRC patients with unresectable primory tumor or metastatic disease [176-179].

Factor	
• *Performance status* (decreasing karnofsky score)	• *Primary tumor location* (right and transverse colon)
• *Distant metastis* (present, especially liver metastasis or peritoneal carcinomatosis)	• *Routine laboratory parameters* (elevated alkaline phosphatase, white blood count, platelets, lactate dehydrogenase or decreased serum albumin and haemoglobin)
• *Number of metastatic sites* (≥ 1)	• *Pretreatment CEA levels* (elevated)
• *Age* (younger)	• *Progression to first-line chemotherapy* (present)

The development of cancer is due to the accumulation of genetic events and a number of molecular markers involved in this process have been examined as new prognostic indicators in CRC. Some examples of these markers and other probable prognostic factors in patients with CRC are shown in Table 5.

Table 5. Probable prognostic factors in colorectal cancer [180-185].

Factor	Good prognosis	Poor prognosis
TPS		*X*
MSI	*X*	
TGFβ mutation	*X*	
Loss of heterozygosity at 18q		*X*
K-ras mutations		*X*
p53 alterations		*X*
High VEGF expression		*X*
High TS expression		*X*

Abbreviations:
TPA = tissue polypeptide antigen, TPS = tissue polypeptide specific antigen,
TS = thymidylate synthase

The prognostic value of soluble cytokeratin markers, such as tissue polypeptide antigen (TPA) or tissue polypeptide specific antigen (TPS) have been limited evaluated in CRC [181]. However, they have shown to be useful in monitoring therapy in patients with advanced disease [186].

Mutations in the p53 tumor suppressor gene with overexpression of its protein product are present in 40% to 50% of CRCs [187]. The relationship between p53 alterations and prognosis in CRC has showed discordant results [180,188].

Likewise, there has been no clear association between EGFR expression and prognosis in CRC. However, that might be due to lack of standardised assays for EGFR-expression [189].

High intratumoral expression thymidylate synthase (TS) appear to predict a poorer survival compared to patients with low expression in patients with both local and advanced CRC [182,190].

3.4 Screening

Screening for CRC with fecal occult blood test (FOBT), and/or sigmoidoscopy works and reduces the mortality [191,192]. Colonoscopy has also been used as a primary

screening test but no study has assessed the efficacy of this method in reducing mortality [193]. New screening test, such as virtual colonoscopy and fecal DNA testing, are being developed [193]. Screening for CRC is not yet introduced in Sweden.

3.5 Treatment strategies

Surgery is the primary curative modality in patients with CRC, and important developments have occurred. Especially in rectal cancer, one of the most significant advances for surgery has been the concept of total mesorectal excision (TME) in rectal cancer [194], which dramatically reduces local recurrences rates [195,196]. Despite the fact that approximately 80% of patients are eligible for curative surgical resection at the time of diagnosis, 40–50% of these patients develop local recurrence or metastatic disease [197]. This is presumably due to the presence of disseminated micrometastases at the time of surgery.

3.5.1 Adjuvant treatment

Colon cancer

The aim of adjuvant treatment in colon cancer is to prevent local recurrence or distant metastases and to prolong survival. For patients with colon cancer stage III, chemotherapy is the principal adjuvant treatment. The predominant drug used has been 5-fluorouracil (5-FU) [198]. During the seventies and eighties, several randomized studies failed to demonstrate a significant survival benefit of chemotherapy until Moertel et al [199] published a positive study in 1990. National Institute of Health quickly made a consensus statement that chemotherapy with 5-FU and Levamisole should be standard therapy in stage III colon cancer. During the nineties several more studies followed also demonstrating the gain in cure by adjuvant chemotherapy [200]. Since the middle of 1990's adjuvant chemotherapy in stage III colon cancer has became a standard option also in Sweden, where a combination of 5-FU/LV for 6 months is used [201]. For these patients the overall reduction in disease-specific mortality is approximately 30% (or an absolute survival benefit of 5–7%) [201]. The value of new agents has been investigated. Capecitabine, an oral fluoropyrimidine derivate, may be considered as an equal alternative to 5-FU/LV [202]. The camptothecin alkaloid derivate irinotecan is active in colon cancer but combined with 5-FU/LV in the adjuvant setting, failed to improve survival as compared with 5-FU/LV alone [203]. On the other hand, oxaliplatin, a third-generation platinum derivate, combined with 5-FU/LV did improve 3-year DFS survival as compared with 5-FU/LV alone [204].

Adjuvant chemotherapy of patients with stage II colon cancer remains controversial [169,205,206]. However, for specific “high-risk” stage II patients (eg; T4 tumor, obstruction or perforation of the bowel, poor differentiation or less than 10 lymph nodes examined from the surgical specimens), adjuvant chemotherapy should be considered [169,204]. Adjuvant chemotherapy in elderly (> 70 years) patients has been found to be as beneficial as it is in younger patients [207]. However, by older age the toxicity increases why chemotherapy in CRC rarely is indicated above the age of 80 [207]. Most studies have used an upper age limit of 75 years.

Other adjuvant approaches in colon cancer

Edrecolomab (monoclonal antibody 17-1A)(section 3.6.3.) has been investigated as an adjuvant treatment for CRC. In a German study, stage III CRC patients were randomized to treatment with Edrecolomab versus surgery alone [130]. After 7 years follow-up evaluation, treatment had reduced overall mortality by 32% and decreased recurrence rate by 23%. Two large randomized multicenter studies have been performed. In the first, Edrecolomab was compared to 5-FU/LV and the combination [208]. Survival figures for Edrecolomab alone were comparable to the Riethmüller study, indicating some effect. However, significantly worse than 5-FU/LV and the combination arm was not better. In the second study, 5-FU/LV was given alone or together with Edrecolomab [209]. In this study the combination was significantly superior. In a third study in stage II disease, Edrecolomab was compared to observation and no significant improvement of Edrecolomab could be found [210].

Rectal cancer

For patients with rectal cancer, adjuvant irradiation (pre- or post-surgery) reduces local recurrences rate, increases overall survival and is standard treatment [211]. Results obtained from randomized trials have indicated that radiotherapy will have an impact on local recurrences even if surgery is optimized [212]. In Sweden, preoperative irradiation, frequently 5 x 5 Gy in one week is established for patients with stage II and III rectal cancer. If the tumor is considered non-resectable (T4) at diagnosis, preoperative radiotherapy to a dose of 50 Gy given with conventional fraction (1.8-2.0 Gy/fraction) with or without concomitant chemotherapy is recommended, aiming to achieve subsequent curative resection by decreasing tumor size.

3.4.2 Treatment in advanced disease

Approximately 20% of all CRC patients have advanced disease at initial diagnosis, and another 20–30% will develop recurrence or distant metastases, despite a curative

resection. In the majority of patients, relapse is diagnosed within the first 2–2.5 years after surgery [169]. Among the sites of metastasis, the liver is the organ most frequently involved (~40–60% of cases), followed by the abdominal lymph nodes (~40%), the lung (~40%) and peritoneum (~30%). Median survival in patients with metastatic disease and without any treatment is 6–8 months [213]. Prospective studies have demonstrated that the use of palliative chemotherapy in patients with advanced CRC improves quality of life, control symptoms, and prolongs survival in comparison with best supportive care [213,214]. From the 1960s until recently, 5-FU was the dominant agent for the treatment of CRC. Response rates varied between 10% to 15% and median survival at 10 months [213]. The objective response rate (tumor size reduced by 50% or more) was improved to approximately 20% when calciumfolinate was added to 5-FU with median survival of 11 to 13 months [201,213,215]. By approving irinotecan (1998) and oxaliplatin (1999), and two monoclonal antibodies, cetuximab (2004) and bevacizumab (2005), considerable improvements has been made in the treatment of patients with advanced CRC. Median survival is increased to 14 to 16 months when either irinotecan or oxaliplatin is added to a 5-FU-based treatment regimen [216,217] with responses rates of around 40%. It appears that exposure to all three drugs (5-FU, oxaliplatin and irinotecan), regardless of the sequence, is key to further extending survival in advanced CRC [218]. Cetuximab has demonstrated a significant effect in irinotecan resistant CRC, both alone and in combination with irinotecan [129]. Bevacizumab has demonstrated a significant effect in first-line therapy. Combined with standard irinotecan 5FU/LV-therapy the addition of Bevacizumab improved overall survival from 15.6 to 20.3 months [134]. By combining all five drugs it seems possible to reach a median survival exceeding two years.

Patients with lung or liver metastases should be carefully monitored as the chance for curative resection may develop since combination protocols achieve high response rates. Resection of metastatic disease can lead to 5-year survival rates of 35–58% [170] or even more by combination of different treatment strategies [219]. An increasing number of patients can be offered this option.

3.6 Rational for immunotherapy in CRC

3.6.1 Immunogenicity of CRC

There is considerable evidence to indicate that spontaneous immune reactions against the disease may take place in CRC. Furthermore, emerging data suggests that the presence of an immune response to CRC may influence the prognosis [220]:

- Natural antibodies against TAAs such as CEA and EpCAM have been reported [221,222] and have been associated with improved survival [221].
- Spontaneous cellular responses against tumor antigens are induced in some CRC patients [223]. This peripheral T-cell response against HLA-A2-binding epitopes of TAAs did, however, not have any prognostic role [224].
- High NK cell cytotoxicity in peripheral blood has shown to correlate positively with prognosis in CRC patients [225] (I).
- The prognostic significance of prominent lymphocyte infiltrates in the direct vicinity of the tumor in CRC patients was showed in 1986 by Jass [226]. Since then several studies have shown the accumulation of effector cells from both the innate and adaptive immune system, in the nearby of the solid tumor in CRC patients.
- The presence of tumor-infiltrating DC [227], NK cells [42,228], eosinophils [220] and TAMS (especially VEGF-expressing) [229] in tumor specimens of CRC have been reported to positively correlate with prognosis.
- More recent studies have shown the tumor-infiltrating leukocyte fraction consist mainly of CD8+ T cells [228], and the NK cell fraction is relatively small. Although the prognostic role of tumor infiltrating CD8+ T cells have been a debated issue, the presence of those cells have been associated with improved survival [31,228] in CRC patients. The location of lymphocyte infiltrates seem to be of importance as only those CD8+ T cells located in the tumor epithelium affected the prognosis positively [31].
- Aberrant MHC class I expression (down-regulation or total loss) have been detected in approximately 70% of CRC patients [51]. In a very recent report including more than 450 CRCs analysed in tissue micro array, down-regulation of MHC class I expression was an independent marker of poor disease-free survival compared to those with high levels or total loss of MHC class I expression [230].
- MSI positive tumors are associated with pronounced lymphocytic infiltration and have better outcome in comparison with MSI negative tumors [185].
- Clinical evidence that immunotherapy may be effective in CRC [88,130,209,231].

3.6.2 EpCAM

The human epithelial adhesion molecule (EpCAM) was first identified by Hilary Koprowski with the murine monoclonal antibody 17-1A (mMAb 17-1A) [232]. It has been described by various other names (CO17-1A, KSA, GA733-2, KS1-4 and EGP) corresponding to MAbs used to identify it in various tissues. EpCAM is a 40 kDa transmembrane glycoprotein that consists of two epidermal-growth-factor (EGF)-like extracellular domains, a cysteine-poor region, a transmembrane domain and a short cytplasmic tail [233]. Both EGF-like repeats form a globular structure and are required for the Ca^{++}-independent, homophilic cell–cell adhesion of EpCAM, but its function is not fully understood. The EGF-like repeats are also required for the anchoring of actin microfilaments at the cell membrane via α-actinin, a process regulated by the cytoplasmic tail of EpCAM [234]. Besides, EpCAM has been shown to be involved in signal transduction and to support cell motility [235]. Overrexpression of the EpCAM gene can also induce upregulation of the proto-oncogene c-myc and support cell proliferation via upregulated synthesis of cyclin A and E [236].

EpCAM is a strictly epithelial molecule in adult humans, expressed on different normal tissues and at a high level by a large number of epithelial derived neoplasias [234]. In CRC, the EpCAM antigen is expressed by more than 90% of all cancer cells on the majority of primary tumors as well as on metastases [237]. Some other epithelial tumors, such as carcinomas of the breast, ovary, stomach and pancreas show intermediate level of EpCAM expression. EpCAM is also expressed on some normal tissue, but the density of antigen expression is much higher on tumor cells [233]. One recent report demonstrated that the expression of EpCAM was significant lower on circulating tumor cells as compared to primary and metastatic tissues [238], suggesting that EpCAM expression may be transient and dependent on the local microenvironment. Normally the EpCAM antigen is not shed in to the circulation, and this may facilitate tumor targeting by MAbs and or T cells [233].

Overexpression of EpCAM, was found to correlate with poor prognosis of patients with gallbladder carcinoma [239] and breast cancer [240]. Loss of EpCAM expression has shown to be associated with decreased survival and the presence of lymph node metastasis in other carcinomas [241]. There is evidence for spontaneous humoral and cellular immune recognition of EpCAM in CRC patients, thereby confirming its immunogenicity in humans [222,223]. Moreover, the generation of EpCAM specific auto-T cells have occasionally been demonstrated in CRC patients [88,224,242] and cytotoxic T-lymphocytes in peripheral blood in colon cancer patients [243] that might indicate that self tolerance towards EpCAM can be broken.

EpCAM has been a hopeful target in immunotherapy with unconjugated monoclonal antibodies [130], active immunotherapy with anti-idiotypic antibodies [242,244] and recombinant protein [88,245].

3.6.3 Edrecolomab

The antibody used in the studies of this thesis was first known as MAb17-1A. Later the name Edrecolomab was introduced. In the adjuvant studies (page 33) the name Panorex was used and the name Adjucal was planned if registered. It has also been referred to as anti-EpCAM MAb. It was among the first MAbs for the treatment of humans with cancer [246]. Edrecolomab binds exclusively to the first extracellular motifs of EpCAM [234], but the binding affinity is relative low (7×10^{-7} M) [247]. The immunological mechanisms of Edrecolomab are proposed to be ADCC, mediated by NK cells and monocytes/macrophages and CDC [114,117]. The induction of an anti-tumor immunity (idiotypic network response) is a third mechanism by which Edrecolomab may exert its effect [101] in vivo.

Clinical effects of Edrecolomab have been demonstrated for CRC patients in the adjuvant setting (page 33) as well as with advanced CRC [88]. Edrecolomab in ajuvant treatment completely eliminated or significantly reduced the number of EpCAM positive disseminated tumor cells in bone marrow (BM) of breast cancer patients [248,249].

In patients with advanced CRC, Edrecolomab given as monotherapy has been shown to induce an overall response rate (including stable disease) in 10–15% of the patients [88,250]. Clinical benefits have been achieved with the addition of cytokines and chemotherapeutic agents, see section 7.

Other unconjugated MAbs in CRC

In addition to edrecolomab, cetuximab and bevacizumab, other MAbs have been developed for treatment of CRC. A fully human IgG1 MAb with intermediate affinity for EpCAM is currently in phase II studies for CRC and other carcinomas [251], and a human-engineered high-affinity MAb (ING-1), recognizing EpCAM, showed low immunogenicity and tolerable toxicity in patients with solid tumors, including CRC [109]. EMD 7200 is a humanized IgG1 MAb that binds with high specificity and affinity to the EGFR [105]. ABX-EGF is a fully human IgG2 MAb that binds with high affinity to the EGFR and to TGF-α [104]. The efficacy of those MAbs in treatment for CRC are identified in ongoing clinical trials.

4 PRESENCE OF CYTOKERATIN POSITIVE CELLS IN THE BONE MARROW OF PATIENTS WITH CRC

4.1 The concept of micrometastases

The traditional definition of metastases, i.e deposits of malignant cells, segregated from the primary tumor, without a specific blood supply with histological prerequisites being implantation, tumor cell arrest and proliferation with a surrounding stromal reaction [252].

The term "micrometastases" seems to indicate that an element of size is involved. Following the suggestion by Huvos et al [253], the UICC decided to limit the size of significant micrometastases in the LN > 0.2mm to ≤ 2mm [166]. In some instances the number of tumor cells detected by immunohistochemistry could be ≤ 0.2mm (approx. 10–15 cells) scattered in the lymph node, i.e. *"Submicrometastases"/- Isolated tumor cells (ITC).* In the AJJC/UICC staging of CRC, the ITC in the LN are to be staged as pN0 (i+) [167] .

When considering the concept of bone marrow micrometastases (BM) of patients from any of the carcinomas where this phenomenon has been demonstrated, no guidelines exit, as to what constitutes BMM. The only accepted convention seems to be based on the presence of cytokeratin positive (CK+) epithelial cells in the marrow. Sloane et al [254] were the first to coin the term "*micrometastases in bone marrow*" after immunocytochemically demonstrating the disseminated carcinoma cells in the BM aspirates of breast carcinoma patients.

As used presently, the term BMM seems to be a misnomer as it has come to denote what in reality are isolated tumor cells (ITCs) in the marrow without fulfilling, not only the size requirements but also the histological prerequisites to classify the phenomenon as micrometastases. These cells would ideally qualify to be comparable to the submicrometastases/ITC in the LN, according to the joint UICC/AJCC staging of CRC [167]. In addition to the LN and BM, ITCs have been described in the blood from CRC patients [255].

4.2 Cytokeratin

Cytokeratins (CKs) form an intracellular network of intermediate filaments proteins, that is believed to play a vital role in maintaining mechanical integrity of and account for almost 85% of the total cellular protein of epithelial cells [256-258].

Based on molecular weight and isoelectric points, at least twenty individual polypeptides, have been characterized, divided into two groups; Type I and Type II, with an overall homology of structure, size, and charge [256,257]. The expression of

CK varies with the type of epithelium and at least two different types of CK, one member from each group, are expressed in any given epithelium [258]. During transformation of normal epithelial cells into malignant cells, the cytokeratin patterns are usually maintained [258,259]. This property of CK is used extensively in the differential diagnosis of tumors in routine histopathology. Thus, for optimal detection of micrometastases, more than one type of cytokeratin would be needed [260].

The expression of CK in the BM seems to be relatively specific for epithelial cells [258]. However, a false-positive rate of < 2% in BM has been reported [260,261]. One of the false positives being a low level of ectopic cytokeratin expression reported in some malignant hematological cells, suggesting a potential for disease-induced false-positive analysis [262].

5 AIMS OF THE THESIS

- To evaluate the clinical effects of the combination of mouse anti-EpCAM MAb and immunomodulating cytokines as well as chemotherapeutic agents in patients with advanced CRC.
- To explore the clinical effects, adverse events, and immunological responses of chimeric anti-EpCAM MAb in combination with GM-CSF.
- To study the prognostic impact of cytotoxic cells in patients with advanced CRC receiving anti-EpCAM MAb as first-line treatment.
- To study the pharmacokinetics, biological and immunological effects of GM-CSF *in vivo.*
- To evaluate if cytokeratin positive cells in the bone marrow can serve as a surrogate end point for assessing therapy in CRC.

6 PATIENTS AND METHODS

Patients

Patients included in papers I–VI had advanced CRC not accessible to surgery and a Karnofsky index of ≥ 80%. In papers V and VI also patients with surgically resected CRC with no evidence of disease (NED), were included. The studies were approved by the Ethics Committee of the Karolinska Institute.

Treatment protocols

Patients with advanced CRC were treated according to various consecutively anti-EpCAM MAb based protocols;

1. Anti-EpCAM murine (m) MAb: Anti-EpCAM mMAb was given intravenously (i.v.) alone in varying schedules and total doses ranges from 1 to 12 g [250,263]. Patients are included in Paper I.

2. Anti-EpCAM mMAb/GM-CSF: Recombinant human (rh) GM-CSF was given subcutaneously (s.c.) for 10 days. At day 3, 400 mg of anti-EpCAM mMAb was infused i.v. [76]. Patients are included in Papers I, III and IV.

3. Anti-EpCAM mMAb/GM-CSF/IL-2: rh GM-CSF was administered s.c. for 10 consecutive days. rh IL-2 was administered simultaneously, s.c. twice daily for 10 days. At day 3 of a treatment cycle, 400 mg of anti-EpCAM mMAb was infused i.v. [264]. Patients are included in Papers I and IV.

4. Anti-EpCAM mMAb/GM-CSF/IFNα/5-FU: rh IFNα was given s.c. for 5 consecutive days. At days 4 and 5, fluorouracil (5-FU) was administered i.v. Following 2 days rest, rh GM-CSF was given s.c. days 8-14. On day 10, 400 mg of anti-EpCAM mMAb was given i.v. The clinical results are reported in Paper II and patients are included in Papers I, IV, V and VI.

5. Anti-EpCAM chimeric (c) MAb/GM-CSF: rh GM-CSF was given s.c. for 10 days. At day 3, 200-400 mg of anti-EpCAM mMAb was infused i.v. The clinical results are reported in paper III and patients are included in Papers I, IV, V and VI.

6. MDX-HER 2/GM-CSF: rh GM-CSF was given s.c. for 14 consecutive days. At days 3, 7 and 11 bispecific antibody (BsAb) MDX-H210 [265] was given i.v. (data to be published). Patients are included in Papers V and VI.

Patients with no evidence of disease (NED) received adjuvant vaccinations or chemo- and/or immunotherapy;

7. Recombinant CEA protein: r CEA protein was given s.c. at weeks 0, 2 and months 2, 4, 6, 9 and 12 with or without rh GM-CSF [151] to patients with AJCC stage I–IV disease.

8. Recombinant EpCAM protein: r EpCAM protein or a human anti-idiotypic Ab (anti-Id) was given s.c. at weeks 0, 2 and 6 with GM-CSF [266] to patients with AJCC stage II–III disease.

9. ALVAC-KSA: Recombinant avipoxvirus (ALVAC) expressing the full-length EpCAM-gene (ALVAC-KSA) vaccine was administered s.c. at weeks 0, 3 and 6 with or without GM-CSF (9) [154] to patients with AJCC stage I–III disease.

10. Anti EpCAM mMAb +/− 5-FU/LV: Anti-EpCAM mMAb was given i.v. alone or in combination with 5-FU and Leukovorin (LV) or 5-FU/LV alone [208] to patients with AJCC stage III colon cancer.

Criteria for response and follow-up

Response evaluation for patients with advanced disease was performed according to complete remission (CR), partial remission (PR), minor response (MR) and stable disease > 3 months (SD) as described in details in paper II and III. Patients with advanced disease were assessed for overall survival (OS) and progression free survival (PFS) from start of treatment until death. Patients with NED were assessed for OS and disease-free survival (DFS) for at least 5 years from start of therapy (median follow-up for patients in treatment protocol number 8 was approximately 4 years) or until death, lost during follow-up or until disease recurrence.

Cytotoxicity test

Cytotoxic activity was determined in an 18h ^{51}Cr-release assay. Briefly, target cells (a human CRC cell line SW948 expressing the EpCAM-antigen [267] and K562 a human chronic myelogenous leukemia cell line [268] were labelled with sodium ^{51}Cr. After washing, the cells were added to round-bottom wells of a microtiter plate and effector cells were added.

Results were expressed as percent lysis at an effector: target (E:T) ratio of 50:1 (I and IV) or as lytic units (LU)/10^6 effector cells (peripheral blood mononuclear cells (PBMC)). In ADCC, anti-EpCAM murine or chimeric MAb was added.

Measurement of GM-CSF

GM-CSF concentration in sera was assayed in ELISA according to the manufacturers' instructions (IV).

Quantitative flow cytometry for GM-CSF receptor (GM-CSFR) expression

The expression of GM-CSFR (α and ß-subunit) was performed by direct immunofluorescence staining using haemolysed fresh peripheral blood leukocytes (PBL) and commercially available MAbs. Phycoerythrin (PE) conjugated goat anti-mouse MAbs were used as secondary antibodies. Cells were then washed and incubated with FITC-conjugated mouse anti-CD14 and anti-CD66b. Irrelevant isotype-matched mouse IgG was used as negative control. Flow cytometric analyses were performed using a FACS (fluorescence activated cell sorter) Calibur flow-cytometer. Monocytes and granulocytes identification was based on their expression of CD14 and CD66b, respectively. For each sample, 10 000 events were aquired for a live gate and per cent of CD14+/GM-CSFRα+, CD14+/GM-CSFRß+, CD66b+/GM-CSFRα+ and CD66b+/GM-CSFRß+ cells were recorded. Cellquest® software was used to determine the mean fluorescence intensity (MFI) for acquisition and analysis. The quantitative level of GM-CSFR (α and ß-subunit) expression were given as the total number of positive cells as well as MFI ratio (IV).

Immunohistochemisty analyses of cytokeratin positive cells in bonemarrow

The following methods have been described in the detection of bone marrow micrometastases (BMM):

A) Density gradient separation of BM aspiration (BMA);

1. Immunocytochemistry (ICC):
   - (anti-EMA MAb) [254],
   - broad spectrum anti-cytokeratin (anti- A45-B/B3 MAb)[257,260].

2. Flow cytometry:
   - (anti- CK 18 MAb) [269].

3. Enrichment techniques:
   - **Positive separation**: immunomagnetic beads coated with MAbs against various CK [270], TAA (CO 17-1A) [271], or bi-specific immunomagnetic beads coated with anti Ep-CAM, and anti Her2-neu [272].
   - **Negative separation** with immunomagentic beads coated with MAbs against leukocyte common antigen (LCA) [273].

4. Molecular methods:
   - RNA: rt-PCR, quantitative rtPCR, using primers to detect CK 20 and CEA [274].
   - DNA: FISH [275].

B) Bone marrow biopsy or clot;

1. Immunohistochemistry: AE1/AE3 **Papers V and VI**.

When the study was first conceived, the lately described molecular methods were in their early years and not yet standardized. Thus, it was decided to use Immunochemistry.

The bone marrow clot technique has been used in routine haematopathology at the Karolinska University Hospital since the early 1980s. The clear advantage of this method is the preservation of the cyto-morphology with preserved intercellular relationship of marrow cells. The main disadvantages of the method are the loss of the histo-morphology, and the more extended time required to stain and evaluate the sections.

Preparation of the bone marrow clot, routine staining and immunohistochemistry

With the aid of a 16-GA disposable "I" type BM aspiration needle, 5 ml of BM was aspirated under local anaesthetic from bilateral posterior superior iliac spines. The clot was then fixed in 4% neutral buffered formalin for 4 hours and processed for paraffin embedding.

8 μ sections were deparaffinized and rehydrated in decreasing grades of alcohol, and stained routinely with H&E, Prussian blue, Gordon Sweet (silver impregnation of reticulin), and periodic acid Shiff's reagent (PAS) respectively. BM clots were stained immunohistochemically for cytokeratin (CK), p53 and Ki67 by the sequential double immunohistochemistry method as described earlier, briefly; For detection of p53 (MAb p53 (Do 1, wild type p53), (1:100), (Santa Cruz Biotechnology, CA, USA), and Ki67 (MAb MIB-1 (Ki 67), (1:150), (a gift from Dr Johannes Gredes, Borstel Institute of Research, Borstel, Germany)), a peroxidase labelled avidin-biotin complex (ABC) method was performed with an elite ABC kit (Vector Lab., Burlingame, CA, USA) (step 1) followed by an alkaline phosphatase labelled streptavidin-biotin complex (Dakopatts, Copenhagen, Denmark) staining of CK (the anti-pancytokeratin MAb AE1/AE3 (1:100) (Boehringer Mannheim, Germany) (step 2).

Statistical Methods

Statistical analyses were performed using SPSS version 11.0 (I, II and III), 12.0 (IV), 13.0 (VI) and statview ® (SAS institute Inc., USA; Third Edition, 1999) software program (I, II, III, IV and VI).

Survival curves were generated by the life table method (I, II, III and VI) and significance was determined by the log-rank test (I, II, III and VI) or Wilcoxon (Gehan) statistic exact test (VI).

The independent significance of each prognostic variable related to OS, DFS and PFS was determined by multivariate analyses using Cox projectional hazards model (I, II and VI). The level of significance was obtained from the Wald statistic. The Chi-square or Fisher's exact tests, wherever appropriate, were used for comparison of distributions between groups (I, II, III, IV and VI).

To estimate the reproducibility of the cytotoxic assays over time in paper I, linear regression was used to estimate the relationship between cytotoxicity and calendar year for each control donor participating once. For each control donor participating twice or more, a regression line for cytotoxicity was plotted against calendar year, using all available observations. The linear slope was then estimated and tested for equality analysis of covariance (ANCOVA).

Analysis of the prognostic impact of response was performed after an observation period of 4 months, as response is "time dependent" in relation to treatment start. The observation period and follow-up period were then held apart, avoiding the "pitfall of overlapping exposure and follow-up periods" [276] (II and III).

Correlation between continues variables were performed using Spearman rank correlation (I, II and IV). Linear multivariate regression analyses were performed for factors significant in univariable analyses (IV).

Comparisons of continuous variables between groups were performed using student's t-test or the non-parametrics Wilroxon rank sum test (Mann Whitney U-test) or Krushall-Wallis (VI) for unpaired data.

For comparison of dependent observations, the non-parametric Wilroxon Signed Rank was applied for paired samples for comparison between baseline and follow-up data (IV). This non-parametric test was used due to the considerable variability of the analysed parameters.

Comparisons of dichotomous factors within patients were performed with the non-parametric McNemar's test for the significance of changes and Paired sign test (III and VI).

To analyse a relationship between survival (OS, DFS and PFS) and the presence of cytokeratin positive cells in bonemarrow, time dependent covariates was used in Cox regression analyses (VI).

All statistical tests were two-sided and used a p value of < 0.05 for statistical significance.

7 RESULTS AND DISCUSSION

PAPER I

During the last decade, developments of immunotherapeutic strategies in general, and antibody based therapies in particular, has become components of some standard cancer treatment strategies. However, there are few data on which patients will respond to MAb therapy and which in vivo effector functions are most important. Such information would facilitate the strategies to improve antibody-based therapy and might help the selection of patients that are likely to have clinical benefit of the treatment. ADCC in considered one of the most important mechanisms in vivo by which tumor cells can be destroyed using MAbs [112,114].

The main mononuclear cell populations responsible for cytotoxic activity are FcγR-bearing NK cells and monocytes/macrophages [106,107,112,114,115]. We have previously demonstrated a favourable correlation between a high ADCC activity and OS in a small patient population with advanced CRC [76], receiving anti-EpCAM mMAb therapy. The aim of this study was to analyse the prognostic significance of the pretreatment cytotoxic capacity of peripheral blood mononuclear cells (PBMC) in an extended patient population with metastatic CRC receiving anti-EpCAM MAb as first-line treatment.

Seventy-three patients with advanced CRC expressing the tumor antigen EpCAM receiving treatment with either the murine or chimeric anti-EpCAM MAb between 1986 and 1998, were included in this study. Patients were defined as having aggressive disease if distant metastases were diagnosed at primary surgery or within six months of primary surgery (n=29). The remaining patients were considered to have less aggressive disease including locally advanced disease at primary surgery, early local relapse, or late relapse irrespective of location (n=44). Prior to therapy, the cytotoxic activity of the patient PBMC was determined in a ^{51}Cr-release assay against two human target cells; the NK-sensitive human erythroleukemia target cell line K562 and the EpCAM-expressing CRC cell line SW948. Patients were considered to have a high or low cytotoxic capability when the cytotoxic value of PBMC was above or below the median of the whole group for the specific test. Overall survival (OS) and progression-free survival (PFS) were related to the cytotoxic capability for each of the four cytotoxicity assays. Fifty-six healthy donors (41 female, 15 male) were used as controls.

The results of the controls were compared over the whole 13-year study period and the reproducibility over time was satisfactory. Unexpectedly, the patients exhibited a significantly higher cytotoxic capability in all four assays compared with healthy control donors. Pretreatment NK cell cytotoxicity (K562) was significantly related to overall survival (OS), progression-free survival (PFS), and response rate.

OS for patients with high and low NK cell cytotoxicity was 71 vs 30 weeks, respectively (p=0.007). Multivariate analysis showed that NK cell cytotoxicity (K562) was significantly related to OS, followed by calendar year for inclusion in the study. Eighteen hour ADCC and 18-h spontaneous cytotoxicity, which mainly reflects cytotoxic capability of monocytes [115], provided no prognostic information, as demonstrated in our previous study [76]. The subgroup of patients defined as having an aggressive disease, exhibited higher cytotoxicity compared to controls in only one of four assays, and the combination of aggressive disease and low NK cell activity was related to poor survival.

This study implicates that pretreatment NK cell activity in vivo, might serve as a predictor for survival and response to anti-EpCAM MAb based treatment. Whether this observation indicates that patients with a good NK cell activity might be more appropriate to respond to this treatment concept, or those patients with a good NK cytotoxicity, irrespective of therapy, have a better prognosis is not known. There are indications that low NK cell activity may be at higher risk for developing certain cancers [41,277], although testing for immune competence in populations at high risk for malignancy has not been routinely performed. The results from this study suggests that preferentially, patients with preserved NK cell activity should be selected to antibody-based treatments, especially if immune-mediated effector functions of the therapeutic MAb, are worth striving for. Additionally, the finding might support the use of agents, which activate NK cell functions to combine with MAbs, such as IL-2 [278].

PAPERS II AND III

In an attempt to develop an effective monoclonal antibody-based therapeutic regimen in CRC, patients with advanced disease have been recruited sequentially to different regimens at our department with the first patients included in 1985.

In the first treatment series, of six consecutive treatment protocols, increasing doses of murine anti-EpCAM MAb (MAb17-1A) alone, were used. From a total dose of one gram divided in four doses to a maximum of 12 gram divided in 24 doses [250,263]. Only one patient had a clinical response (SD 25 weeks) in the high dose protocols (total dose > 2 gram) indicating that moderate doses might be the most favourable, see Table 6.

Table 6. Tumor response, median progressive free survival (PFS) and overall survival (OS) in patients with advanced colorectal carcinoma (CRC) treated with different regimens containing anti-EpCAM MAb.

Treatment protocol		No. of patients	Response category (n) (%)				Overall response rate		PFS	OS	Ref.
Number	Regimen [a]		CR	PR	MR	SD	(n)	(%)	(weeks)	(weeks)	
1a	Anti-EpCAM mMAb alone <2g	46*	–	1 (2%)	3 (7%)	6 (13%)	10	(33%)	8	43	[250, 263]
1b	Anti-EpCAM mMAb alone >2g	26	–	–	–	1 (4%)	1	(4%)	5	50	[250, 263]
2	Anti-EpCAM mMAb/GM-CSF	22	2 (9%)	–	1 (5%)	3 (14%)	6	(27%)	11	46	[76]
3	Anti-EpCAM mMAb/GM-CSF/IL-2	20	–	1 (5%)	–	2 (10%)	3	(15%)	8	36	[264]
4	Anti-EpCAM mMAb/GM-CSF/ α-IFN/5-FU	27**	–	1 (4%)	2 (8%)	11 (42%)	14	(54%)	15	75	Paper II
5	Anti-EpCAM cMAb/GM-CSF	24	–	–	–	5 (21%)	5	(21%)	9	59	Paper III

a = m=mouse, c=chimeric
b = For abbreviations and criteria see page 49 and Papers II and III
* = number of pts evaluable for response=45
** = number of pts evaluable for response=26

Immune-mediated antitumor mechanisms by unconjugated MAbs might be improved by the simultaneous administration of cytokines. In the subsequent series, anti-EpCAM mMAb was combined with GM-CSF (Protocol 2) [76] and both GM-CSF and IL-2 (Protocol 3) [264].

Based on preclinical in vitro results, our previous clinical using anti-EpCAM mMAb together with GM-CSF, and an animal model [76,88,279], a protocol of α-IFN/5-FU/GM-CSF and anti-EpCAM mMAb in patients with advanced CRC was formed. The aim of this study **Paper II** was to evaluate the clinical efficacy and toxicity, of this combination.

Twenty-seven patients with metastatic CRC not eligible for surgery entered the study. One patient achieved a PR and 13 patients had MR+SD (overall response rate 54%) (Table 6), indicating superiority to MAb17-1A as monotherapy as well as for the combination adding only GM-CSF [76,250]. The addition of α-IFN, 5-FU and GM-CSF to anti-EpCAM mMAb improved PFS compared to patients treated with anti-EpCAM mMAb alone, and although the OS was longer, the difference was not significant. The improved clinical efficacy might not be due to the introduction of new chemotherapy regimens and a better supportive care during the study as the year for inclusion was not a prognostic factor. Additionally, it is not likely that α-IFN and 5-FU alone contributed to the overall anti-tumor activity as the dose of α-IFN and 5-FU in the present study was less than the half of that used when combining α-IFN and 5-FU for therapy of CRC patients [69].

In the majority of patients, side effects were of NCIC grade II or less. No overlapping toxicity was observed with this protocol, and the adverse effects considered to be related to the cytokines, were similar to those of each cytokine. Non-allergic reactions of anti-EpCAM mMAb were mild and comparable to anti-EpCAM mMAb as monotherapy [250].

Due to allergic reactions, the planned anti-EpCAM mMAb dose had to be reduced by repeated infusions. One patient developed a grade III bronchospasm, otherwise the most common types of systemic allergic reactions were flush, urticaria, chest pain, allergic rhinitis and chills. This protocol induced, in contrast to anti-EpCAM mMAb used as monotherapy [130,208,250], allergic reactions in the majority of patients by increasing number of infusions. One explanation to this increased immunogenecity of the therapeutic MAb might be related to the augmentation on APCs of GM-CSF [75].

Our data suggests the clinical benefits of combining anti-EpCAM MAb with other agents with different modes of action. Combination chemotherapy regimens has more recently shown to exhibit different immunomodulating effects on tumor cells lines in vitro [280], in animal models [281], and in patients with advanced CRC [282]. The optimal dose and schedule for 5-FU for immunomodulation in humans is not known and warrants further investigations.

The aim of the study reported in **Paper III** was to analyse the clinical effects and safety of the chimeric anti-EpCAM MAb (MAb17-1A) in combination with GM-CSF. To analyse a possibly dose-response relationship, the GM-CSF dose was altered in some of the patients (**IV**), otherwise the MAb and GM-CSF was given in the same schedule as in Protocol 2. Twenty-four patients were included in this study. Twenty-two patients treated according to Protocol 2 [76] were included in this study as a historical control group.

Five patients had SD > 3 months (overall response rate (21%) with median response duration of 21 weeks (range 18–25 weeks). The median PFS, from start of therapy was 8.5 weeks (range 4–25 weeks) and median OS time 59.5 weeks (range 7–193 weeks) (Table 6). Similar to results in **Paper II**, there was a statistically significant longer OS time for responding patients, as compared to non-responding.

All patients received the planned dose of anti-EpCAM cMAb at the first cycle. Due to type allergic reactions, adjustments of the dose were done at the subsequent cycles.

This study showed, in contrast to what was expected based on in vitro data that the therapeutic effect of anti-EpCAM cMAb and GM-CSF was not further improved by replacement of the murine MAb to the chimeric variant. The Ab_2 concentration [101] as well as the frequency of patients mounting an Ab_2-response was significant lower compared with patients treated with murine MAb. The addition of GM-CSF to anti-EpCAM mMAb has shown to significantly enhance the induction of Ab_2 as well as frequency of Ab_3, compared to anti-EpCAM mMAb alone [101,283]. This might explain the higher incidence of patients mounting an Ab_2 response in this trial (69%) compared to 10% reported previously for this anti-EpCAM cMAb [103]. The frequency of patients mounting an Ab_3 response was similar in the present chimeric trial as in the anti-EpCAM mMAb treatment group (60% and 75% of the patients, respectively).

In summary, although the anti-EpCAM cMAb had a longer half-life [102,103], mediates a higher ADCC in vitro than its murine counterpart [88,284], and is less immunogenic [101,125] allowing a higher dose-intensity (**III**), the clinical outcome did not improve when applied in the corresponding protocol.

PAPER IV

Cytokines, growth factors and other recombinant proteins have all emerged as important components, used alone or in combination with other therapeutic agents, for therapies of an increasing number of malignancies. Recombinant GM-CSF is currently in testing both in combinations with MAbs as well as therapeutic vaccines for human cancers, including CRC [70,73,74]. It is of outmost importance to better understand the dose-response relationship for augmentation of immune functions as

GM-CSF also might induce immune suppression by various mechanisms [73,80]. The dual effects of GM-CSF might be related to the administered dose. Except for a few reports [81,285], data regarding GM-CSF mainly concerns the pharmacokinetics after a single-dose.

The aim of the study reported in **Paper IV** was to establish an optimal therapeutic dose (OTD) and schedule of E. coli-derived GM-CSF (molgramostim) to induce maximum cytotoxic activity as well as to analyse pharmacokinetics of repeated s.c. administration of molgramostim in patients with advanced CRC treated with anti-EpCAM MAb based therapy. Fifty patients with metastatic colorectal carcinoma (CRC) receiving anti-EpCAM MAb based therapy in combination with GM-CSF according to Protocol 2 [76], Protocol 4 (**II**) or Protocol 5 (**III**) were included in this study. Thirty-three patients received a GM-CSF dose of 200-250 μg/m^2/day. Seventeen patients received GM-CSF doses varying between 65-325 μg/m^2/day in the different treatment cycles. Serum GM-CSF concentration was measured (ELISA) before, and 3–4 hours after (corresponding to the peak serum concentration [79,81]) the GM-CSF administration days 1, 5 and 10.

No patient in the present study had detectable GM-CSF levels in serum prior to therapy. There was a significant correlation between the dose of molgramostim and the peak serum concentration of GM-CSF. Following the repeated daily administrations, the peak serum concentration of GM-CSF decreased gradually on days 5 and 10 compared to day 1 in all treatment cycles, and more pronounced in the high dose group. This pattern has been reported for other GM-CSF products as well as other cytokines [81,286].

A dose-dependent increase in total WBC count during the 10 days treatment was observed. Platelet counts and serum albumin concentration decreased, but returned to pre-treatment levels before the subsequent cycle.

Unexpectedly, ADCC of PBMC decreased at days 5 and 10 compared to baseline in the first treatment cycle and an inverse correlation between the dose and cytotoxic index ADCC was noted.

The total numbers of GM-CSF receptor (α-subunit) expressing cells (peripheral blood leukocytes) (PBL) increased significantly during treatment while a transient decline in expression intensity was observed at day 5.

No GM-CSF binding antibodies were seen in 19 tested patients before therapy. At the end of cycle III or IV, approximately 73% had developed GM-CSF-binding antibodies [82]. No patients showed induction of neutralizing GM-CSF antibodies.

Due to the decline in peak serum GM-CSF over the 10 days during treatment with concomitant increment of WBCs, transient decline in GM-CSFR-expression on receptor-bearing cells which seemed to recover, and an inverse correlation between peak serum GM-CSF concentration and total mononuclear cells before GM-CSF injection, this study suggest a receptor-mediated removal of GM-CSF as one

mechanism for the elimination of GM-CSF from circulation. Importantly, this study showed a decline in ADCC of PBMC day 10 of treatment as compared to day 1. The reason for the decline in ADCC is unclear, but it may be related to release of immature cells or induction of suppressor cells. While there seems to be a linear relationship between proliferation and the expansion of cells to the dose of GM-CSF, this might not exist for functional activation [78,79]. High doses of GM-CSF might induce immune suppression by activating monocytes that produce immune suppressive factors [80].

The results indicate that lower doses of GM-CSF would preferentially be used, than administered in our trials, although the optimal OTD of GM-CSF to achieve maximum immune augmentation not could be identified.

PAPERS V AND VI

These studies were designed to study the feasibility of using bone marrow micrometastases (BMM) as a surrogate marker to monitor the adjuvant therapy in CRC and if BMM could be used to earlier assess the effect of treatment in advanced disease, **Paper VI**. In order to achieve these aims, a new method was developed to detect and analyse the BMM, **Paper V**. Fifty-six CRC patients, 34 patients with no evidence of disease (NED) and 22 patients with advanced disease (AD) were included in the methodological paper.

On H&E stained sections, the BMs were either hypercellular or normocellular. There was no morphological evidence of abnormal cells. Except for plasma cells and macrophages, no other PAS positive cells were noticed. In our study 14 of the 56 patients had a local histological reaction to the presence of the CK+ cells in the BM. This reaction consisted of an activated myelopoiesis with small lymphoid follicles and occasionally mild diffuse infiltration of lymphocytes (reactive changes), no desmoplasia was observed. Cytokeratin positive (CK+) cells were immuno-histochemically divided into three groups: Group A (CK+, p53+ and/or Ki 67+, probably malignant epithelial cells), Group B (CK+, p53-, Ki 67+/-, morphologically non-epithelial cells) and Group C (CK+, p53- and Ki 67-, contaminating cells). Plasma cells showed a diffuse homogenous/vacuolated CK expression, and Ki67- as well as p53- nucleus with an occasional cartwheel pattern of nuclear chromatin**.** Both the Group C cells and the plasma cells were disregarded while enumerating the frequency of all types of CK+ cells and therefore not included when referring to CK+ non-epithelial cells (**V**).

The only other study showing various types of CK+ cells in the BM is by Borgen et al [287] who, based on single staining, classify the various types of CK+ cells, into tumor cells (TC), haematopoetic cells (HC), contaminating cells and probable tumor

cells (probable TC). Most likely their tumor cells correspond to our Group A cells, and "plausibly" our Group B cells "correspond" to their probable tumor cells. In our study, due to double staining, we were able to eliminate the false positive haematopoetic cells (**V**).

One-hundred four patients were included in the study reported in **Paper VI.** Sixty-five patients, with NED, received adjuvant treatment. Thirty-nine patients had AD. Bone marrow aspiration (BMA) was examined before, during and after the start of treatment.

During treatment, there was a constant transiting of CK+ cells in the BM of an individual patient. When both the two groups of patients were pooled together, 11 of 68 patients (16%) converted from CK+ to CK neg. while in 9 of 68 patients (13%), the reverse effect was noted and, in 48 of 68 patients (71%) no effect was observed. A similar phenomenon has been reported in breast cancer patients and in pancreatic carcinoma patients [288,289]. While this turnover of CK+ cells in the BM is taking place, the patient clinical status seems to be unaltered, even when the patient shows response to therapy as seen in the AD group, the CK+ cells seem to persist it the marrow. When individual groups were considered separately, the frequency of patients with CK+ cells decreased in the NED group, however, not statistically significant, while no change was seen in the AD group. A comparison of pre-treatment BMA with the last follow-up aspiration, showed that in 13% of patients a positive effect was seen, in 5% a negative and in 82% no effect. In both, univariate and multivariate analysis, patients with presence of CK+ cells did not show any statistically significant adverse prognosis as compared to CK neg. patients. Our results are contrary to that of others [290,291], since we failed to demonstrate a predictive value of BMM in both overall survival and disease free survival in patients with no evidence of disease. It should be pointed out, that there is a difference in our methodology in that our BM samples were collected approximately 6 weeks after curative surgery while Lindemann et al [291] collected the BM prior to surgery and our technique of detecting BMM also varies considerably. However, O'Connor et al [269] using different method to detect CK+ cells concluded that BMM was not predictive of tumor recurrence in CRC. On the other hand**,** the detection of disseminated tumor cells or ITC in peripheral blood has shown to be of prognostic value in patients with CRC [255].

Unexpectedly, the presence of Group A cells (epithelial cells with a malignant morpohology) did not adversely affect the prognosis while the presence of Group B cells (non-epithelial CK+ cells) probably indicates a poor prognosis in the NED group only. This is contrary to that demonstrated by others in breast cancer patients, where the presence of TC (probably corresponding to our Group A cells), showed a worse prognosis [292]. The presence of Group B cells in the BM could represent an in vivo state of the phenomenon reported in vitro [293]. The hypothesis postulated by

Thiery is that the transformation of epithelial cells to mesenchymal cells, i.e. the dedifferentiation of cancer cells towards more primitive cells may lead to a more malignant state. It should be mentioned that the number patients were too few and the *p* value was significant for OS and a trend for DFS.

Multivariate Cox regression analysis for the NED group showed that elevated CEA and/or CA 19–9 levels at pre-treatment BMA predicted significantly for OS (n=59, *p*=0.037). The presence of either CK+ cells or Group A cells in pre-treatment BMA or AJCC stage were not significant factors for OS. In the AD group, if BM was positive for Group A cells then CEA levels were raised while the raised levels of CEA and/or CA19–9 was a poor prognostic factor for overall survival.

In summary, double immunohistochemical staining using CK along with p53 and Ki67 on a routinely processd BM clot, allows the discrimination of various types of CK+ cells. Sequential BM aspirations shows that as these cells are continually transiting through the marrow, using these cells to predict the outcome of therapy or to stratify a treatment based on their presence seems to be a futile effort. This study also demonstrates that BMM seems not to be an additional marker than the existing methods to follow the effect of therapy in CRC. Multivariate analysis of pretreatment CK+ cells in the BM could not be related to prognosis. Occasionally the presence of CK+ cells can predict the recurrence or progression of disease prior to the existing clinical parameters.

8 SUMMARY AND CONCLUSION

Colorectal cancer (CRC) is a public health problem and constitutes the third most common malignancy worldwide. Although extensive advances in the treatment of patients with CRC have emerged in the past years, half of them are expected to die from their disease. In the beginning of 1990s, there was mainly one drug, 5-FU, used for the treatment of CRC with modest clinical efficacy.

In an effort to develop a complementary treatment regimen in metastatic disease, patients have been treated according to different monoclonal antibody-based protocols with anti-EpCAM MAb (MAb 17-1A).

The objectives of the studies presented in this thesis was to evaluate clinical effects and side-effects of two different treatment regimens; anti-EpCAM mMAb/GM-CSF/5-FU/α-IFN and anti-EpCAM cMAb/GM-CSF, respectively, in patients with advanced CRC. To analyze the cytotoxic capability of effector cells as a prognostic and predictive factor. Furthermore, to establish an OTD of GM-CSF. Additionally, the presence of cytokeratin positive cells in the bone marrow of patients before treatment and following therapy in the adjuvant setting or for metastatic disease was studied.

The main findings of the thesis were:

- A significantly higher cytotoxic capability of mononuclear cells was seen prior to therapy in the patients as compared to healthy controls.

- Pre-treatment NK cell cytotoxic capacity was an important predictor for prognosis in patients with metastatic CRC treated with anti-EpCAM MAb as first-line therapy.

- Addition of GM-CSF, α-IFN and 5-FU to anti-EpCAM mMAb increased the response rate and progression-free survival compared to anti-EpCAM mMAb alone in historical controls.

- In contrast to expected from *in vitro* data, the clinical effect of anti-EpCAM MAb in combination with GM-CSF did not further improve when the murine variant was replaced by the chimeric counterpart. The non-allergic reactions of anti-EpCAM cMAb were comparable with those of anti-EpCAM mMAb. Anti-EpCAM cMAb could be administered at higher doses than anti-EpCAM mMAb.

- The frequency of patients mounting an anti-idiotypic antibody response was significantly lower in anti-EpCAM cMAb treated patients as compared to those treated with anti-EpCAM mMAb.

- Following repeated daily s.c. administrations of GM-CSF, the peak serum concentration of GM-CSF decreased significant over the days in a 10-day treatment cycle.

- An inverse correlation between the dose of exogenous non-glycosylated (molgramostim) GM-CSF and ADCC was observed during a 10-day treatment cycle, i.e. the higher GM-CSF dose the lower ADCC activity day 10.

- Various types of cytokeratin positive cells were observed in the bone marrow of CRC patients.

- The presence of cytokeratin positive cells in the bone marrow of patients with radically resected CRC or with advanced disease, showed no impact on prognosis.

Data from this thesis indicates that patients with large tumor burden, several metastatic sites, and/or hepatic involvement of the disease, most likely do not respond to immunotherapy according to this approach. Preferentially patients with a low tumor burden or minimal residual disease should be considered for MAb based therapy. Additionally, patients with a preserved immune system might be chosen.

The antitumor activity of anti-EpCAM as single agent in solid tumors, seemed to be modest, especially in advanced disease, despite proven localization to the tumor area [122,294]. The reason for this is unclear, but it might reflect disturbances in the interactions between the infused MAb, the tumor cells and the immune effector cells of the host. Tumor penetration of IgG is driven by passive diffusion, which might be decreased by a high intratumoral pressure. At the same time, NK cells must reach the antibody-coated tumor cells to achieve ADCC. Although a dense infiltrate of inflammatory cells often are seen in the vicinity of the tumor cells in patients with CRC, NK cells constitute a minority of the tumor infiltrating leukocytes. Additionally, anatomic barriers might hinder the interaction [228].

The addition of cytokines and chemotherapeutic agents with different modes of actions augmented the clinical efficacy of MAb therapy.

The results of this thesis could be of importance for increased understanding of immune functions and for the further development of combinations of cytokines, MAbs and chemotherapeutic drugs for treatment.

9 FUTURE PROSPECTS IN IMMUNOTHERAPY WITH MONOCLONAL ANTIBODIES

Since Köhler and Milstein described the hybridoma technology for production of murine MAbs in 1975, recently developed technologies have enable the selection and identification of chimeric, humanized and fully human antibodies, as well as the improvement of MAb affinity [106]. Intense research of the molecular mechanisms of cancer cell proliferation has revealed that neoplastic transformation is a multistep process, involving changes in characteristics "hallmarks" of cancer [161]. With an increased understanding at each level of defects, new targets have been identified, against which "targeted therapies" have been developed. This increased knowledge has resulted in that antibody-based therapeutics have emerged as important components of therapies for an increasing number of malignancies, especially haematological malignancies, but also for solid tumors. In combination with standard chemotherapy regimens, bevacizumab significantly prolongs the survival of patients with metastatic CRC. Cetuximab used alone or with chemotherapy, induces clinically meaningful anti-tumor responses in patients with chemo-refractory CRC.

Chimeric, humanized and fully human MAbs are supposed to interact more efficiently with human effector functions, and are in clinical use. Other approaches to increase the clinical efficacy of MAbs include the production of bispecific antibodies and fusion of antibodies with cytokines [89].

Based on current data, it appears that the efficacy of therapeutic MAbs seems greatest when they are combined with standard cytotoxic agents. However, several questions remain to be addressed in those combinations. Which combined treatment regimens obtains the largest clinical benefits, and with tolerable toxicity? What sequence and timing of the combination strategies will result in the best clinical outcome for the patients?

Today there are quite a few studies supporting the immunomodulating effects of standard chemotherapeutics used for treatment of CRC. In the future, we will probably see preclinical and clinical trials evaluating this topic [282].

As ADCC seems to be an effective mechanism of action of MAbs, efforts should be made to augment the cytotoxic capability of the effector cells. Cytokines, such as α-IFN, IL-2 and GM-CSF seems to be candidates for such an augmenting effect, and trials are ongoing [278,295]. However, the dual effects of certain cytokines, such as GM-CSF, able to either enhance or restrain the immune response in a dose-dependent way, must be considered. It is of great importance to find an optimal biological dose, which is not the same as maximum tolerable dose. The optimal dosing and scheduling of cytokines based on measurements of GM-CSF in serum seems to be difficult, due to the large variations in serum concentration in humans receiving the

same dose. This requires identification of new biological variables and markers of GM-CSF-induced immunological effects.

Selection of the appropriate patient population is of great importance. Likewise, there is an urgent need to find predictive factors. Patients with a preserved immune status might have a better chance to respond to immunotherapeutics. The identification of immunological parameters, as well as other predictive factors, might help selecting the most appropriate patients, to treat patients that have a chance to have clinical benefit of therapy, and to avoid treating patients that may not have the benefit.

Many studies reported that combinations of molecular markers could be useful in predicting the outcome for patients with CRC. Some of these markers may be useful to identify high-risk categories of patients, who might benefit for adjuvant therapy [180,185].

New assays, such as gene expression profiling [296] or proteomics, will probably be of major interest in the future, to identify important differences between normal and malignant cells. They will help to improve the identification of novel targets for immunotherapy. Ideally, the target antigen should be tumor specific, strongly immunogenic and possibly has a relevant role in tumor cell formation, growth, and progression. A better knowledge of the interactions between the target structure and the MAb, and identification of crucial effector mechanisms in vivo of the MAbs is of importance for further development of immunotherapy.

To develop effective therapeutic strategies against minimal residual disease, standardized and reproducible techniques that can detect and quantify metastatic cells would be of importance to improve the prognosis. The importance of disseminated tumor cells (DTC) in the BM has shown contradictory results. It may be worthwile to focus on the detection, quantification and monitoring of DTC in blood. As the DTC have been shown to have heteregenous characteristics it may be prudent to use multiple markers against various CK known to be expressed in a given carcinoma, possibly by multiplex rt-PCR technique [297]. In CRC, the primers might be constituted to detect e.g. CK 7, CK 18, CK 19 and CK 20.

Using a combination of MAbs with different target antigens, and possibly also different effector mechanisms, may further improve the efficacy of non-conjugated MAbs in solid tumors. Future clinical trials will demonstrate whether a combination of MAbs against different antigens or MAbs against a specific antigen may obtain additive or synergistic effects [298].

Active specific immunotherapies in CRC have mainly been performed as phase I/II nonrandomized studies in patients with advanced disease. In the next 5 years, data from ongoing phase III studies will be available [74], and new generation vaccine candidates may be investigated. A future senario might be to initiate treatment with

monoclonal antibodies which is then followed by maintenance therapy with vaccine treatment.

Immunotherapy against human malignancies is a rapidly developing field. Both preclinical and clinical evidence demonstrate that colorectal cancer might be an immunogenic tumor, thus justifying further research on the development of immunotherapy against CRC.

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