Part 1

Cancer and the Immune System
Cancer is a word that refers to approximately 150 diseases that exhibit two characteristics in common: (1) an uncontrolled growth of cells and (2) the ability to invade and damage normal tissues either locally or at distant sites in the body. Cancer incidence is rising are expected to get some or other form of cancer in their lifetime. Cancer occurs when the DNA present in a gene is altered in such a way that the gene can no longer instruct the cell to produce specific proteins in the normal manner. Such alterations take place when a gene is exposed to radiation or particular drugs or chemicals that bring about damage DNA within a gene ensued by its breaking and incorrect recombination., ultimately leading to various mutations. These factors may not only transform normal gene into oncogenes, but also inactivate antioncogenes leading to full blown tumour.

It is indisputable that some degree of immune response against cancer exists in animals and humans. Components of the immune system that are capable of recognizing cancer cells have been identified in patients with certain cancers. In the laboratory, cells of the immune system can kill tumor cells. Even more convincing are clinical results showing that stimulation of the immune system with bacterial products or components of the immune system itself can lead to tumor regression in some patients. The link between cancer and the immune system is also suggested by the fact that people with an impaired immune system, such as AIDS patients, are more likely to develop certain cancers, including Kaposi's sarcoma, rectal cancer and some specific types of lymphomas (Kiessling et al, 1999).

1.1 Immune response modifiers

These are substances, either extrinsic or intrinsic to the body, that affect the immune response. One group of extrinsic modifiers is referred to as immune potentiators. These include BCG, Corynebacterium parvum and endotoxin, which are all microbes or microbial products that have been shown to modify the immune response and, under certain conditions, to cause tumors to regress or grow more slowly than usual. The intrinsic group, known as biological response modifiers, includes interleukin-1 and interleukin-2, interferon (alpha, beta, and gamma), tumor necrosis factor (TNF), B-cell growth factors and hematopoietic growth factors (such as colony-stimulating factors).
These agents exert their influence at different stages of the immune response. (Daemen et al, 1986)

The interleukins activate the body's own lymphocytes to do their work. For example, IL-2 has been found to be effective in some patients with melanoma or with renal cancer when it is administered alone or with a patient's own lymphocytes that have been treated with IL-2 outside the body (Maass et al, 1995).

The interferons act on the immune system by stimulating both T cells and macrophages. They also prevent cells from multiplying. Scientists believe that these two properties together enable interferon to fight some tumors effectively. Alpha interferon was the first FDA-approved biological response modifier for the treatment of cancer (it is effective against a rare form of leukemia) (Kiessling et al, 1999).

Tumor necrosis factor directly attacks and kills tumor cells. Currently, it is being tested alone as in conjunction with gamma interferon to determine its potential efficacy in the treatment of human cancers. B-cell growth factors stimulate the multiplication of antibody-producing cells. The hematopoietic growth factors step up the production of both red and white blood cells in the bone marrow, thereby giving the body additional ammunition to fight disease and protect itself against the suppressive effects on the bone marrow of radiation and chemotherapy. (Old and Chen, 1998)

1.2 Activation of macrophages

The macrophages are involved in many different processes such as tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, haemopoiesis and homeostasis. Another function of macrophages is to provide a defense line against microbial invasion and to recognize and kill tumor cells. Macrophages can accomplish this in a direct manner, involving the release of products such as oxygen radicals and tumor necrosis factor that are harmful to microorganisms or cancer cells. On the other hand, they play an indirect role in such anti-microbial or anti-tumor activities by secretion of cytokines or by antigen processing and presentation, thereby regulating the immune system. In contrast to macrophage involvement in constitutive processes, macrophage participation in host defense against tumor cells requires activation signals. Activation of macrophages proceeds via different stages
accompanied by gradual changes in macrophage properties. Based on the differences in capacities and functions, macrophages can be characterized as unstimulated, primed and fully activated macrophages (MacKay et al, 1986). Each stage is accompanied by specific expression of membranous and secreted proteins, which can be down- or up-regulated when macrophages develop into another activation state. Changes in protein expression often correlate with the functional ability. For example, for antigen presentation to T-cells, macrophages are dependent on MHC class II molecules. These molecules are lowly expressed on unstimulated macrophages, but are highly expressed on primed, and to a lesser extent on activated macrophages. Hence, antigen presentation is maximal in the primed state. Similar correlations are found for the release of proteins responsible for tumor cell killing, such as TNF (Adams et al, 1992). Since macrophage activation is regulated by both inductive and suppressive signals, activated macrophages can switch from the activated state to the responsive state. One can distinguish two major categories of biological response modifiers that are able to activate macrophages to tumor cytotoxicity.

1.2.1 Bacterial cell wall constituents

First of all, macrophages can be activated to tumor cytotoxicity by components and products of microorganisms. Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, is one of the most potent activators of macrophages and monocytes. However, in vivo LPS may cause harmful effects such as hypotension, fever, disseminated blood clotting and even lethal shock (Rietschel et al, 1992). The mechanism by which LPS is able to activate macrophages is not fully understood, but there is evidence that in plasma a protein referred to as LPS-binding protein binds to LPS. The LPS–LPS-binding protein complex is able to bind to CD14, which is expressed on the surface of macrophages (Wright et al, 1990). Binding to CD14 and possibly of additional surface molecules is followed by signal transduction, which subsequently may lead to the activation of LPS-dependent genes (Wright et al, 1990). Until recently little was known on how the LPS signal is transduced across the plasma membrane as CD14 lacks an intracellular signaling domain and therefore probably does not trigger intracellular signaling directly. Several groups demonstrated that members of the Toll-like receptor family, which resemble the IL-1 receptor family (Medzhitov et al, 1997),
play a role in this phenomenon. Of the Toll-like receptor family, especially the Toll-like
Receptor 2 seems to support an important role in the macrophage responsiveness to
bacterial LPS (Kirschning et al., 1998; Yang et al., 1999). Analogues of lipid A, such as
monophosphoryl lipid A, are less toxic than the parent molecule but have also numerous
effects on macrophages, such as induction of TNF-α IFN-γ and induction of nitric oxide
synthase (NOS), etc. However, the less toxic analogues of LPS and lipid A are in general
less potent in activating macrophages than the parent compounds (Hattori et al., 1995;
Kiener et al., 1988).

The cell wall of gram-positive bacteria does not contain LPS, but instead contains
peptidoglycans that have properties similar to those of LPS. Muramyldipeptide (N-acetyl
muramyl-alanyl-isoglutamine) of mycobacterium has been shown to be the
minimal structural entity of the cell wall capable of inducing adjuvant activity,
modification of non-specific resistance to infections and tumors and activation of
macrophages (Daemen et al., 1986). Many analogues and derivatives of
muramyl-dipeptide have been synthesized for potential clinical use such as murabutide
(Darcissac et al., 1996) and the lipophilic derivative muramyltripeptide
phosphatidylethanolamine (MTP-PE) (Barratt et al., 1994; Hoedemakers 1993). The
effects of muramylpeptides but also those of LPS and its derivatives on macrophages
include upregulation of cytokine production (TNF α, IFN-γ, interleukin-1 (IL-1)),
expression of inducible NOS (iNOS), nitric oxide (NO) secretion, as well as adhesion
molecules. All these processes can lead, directly or indirectly, to increased cytotoxicity of
the macrophages.

In contrast to LPS, muramyl-dipeptide and MTP-PE can be injected safely in vivo.
However, muramyl-dipeptide, due to its low molecular weight and water solubility, has
the disadvantage of being rapidly excreted from the body without exerting an
immunotherapeutic effect. When incorporated into particulate drug carriers, the retention
time in vivo of both muramyl-dipeptide and MTP-PE is significantly prolonged (Fogler et
al., 1985). A major advantage of using such drug carriers to incorporate macrophage-
activating compounds is that, after i.v. or i.p. injection, the drug, together with the carrier,
is primarily taken up by macrophages (Fogler et al., 1985). The macrophage activating
agents are thus passively targeted to the macrophages.
1.2.2 Macrophage activating cytokines

The second category of macrophage activating agents are cytokines. Macrophage activating cytokines predominantly produced by activated T-lymphocytes are generally referred to as macrophage activating factors which include IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) as the most important ones. The activation of macrophages by these cytokines requires the binding of the cytokine to a receptor on the surface of the macrophages which will subsequently lead to the activation of an intracellular signaling pathway (Yagisawa et al, 1999) and ultimately leading to modulation of the macrophage function.

IFN-γ has been studied extensively as an activator of macrophage functions (Drapier et al, 1988; Green et al, 1992). Incubation of macrophages with IFN-γ enhances the respiratory burst, the NO secretion, IL-1 secretion and anti-tumor cytotoxic activity. In addition, IFN-γ enhances receptors and cell surface markers such as adhesion molecules and major histocompatibility complex (MHC) class II antigens (Cavaillon et al, 1994).

GM-CSF induces proliferation of granulocyte and monocyte precursor cells (Ulich et al, 1990), while M-CSF controls the proliferation of monocyte/macrophage precursors and differentiation of monocytes. Both agents effect macrophage functions, which ultimately can lead to tumoricidal activity. Both GM-CSF and M-CSF increase the cytokine production of macrophages and antibody-dependent cellular cytotoxicity (ADCC). In addition, GM-CSF augments the expression of adhesion molecules, respiratory burst and the phagocytotic capacity.

Depending on the way of administration and nature of cytokine, the half-life varies from 20 min to 1 h after i.v. administration and is prolonged for several hours after s.c. administration (Hovgaard et al, 1992). Comparable with bacterial compounds, cytokines have also been incorporated into drug carriers, resulting in a 10–20-fold prolongation of plasma half-lives compared to soluble cytokines (Kedar et al, 1997).

In summary, both bacterial cell wall components as well as certain cytokines can activate monocytes and macrophages to tumor cytotoxicity. Although several effects on macrophage activation are similar between cell wall components and cytokines, also differences in activation patterns between both groups of agents are observed. For
example, induction of nitrogen oxide intermediates is mainly observed by bacterial cell wall components while, on the other hand, some cytokines augment for instance antibody dependent cellular cytotoxicity. These differences in effects are caused by the differences in receptors bound by these agents leading to activation of different signal-transduction pathways.

1.3 Mechanisms of tumor cell killing

1.3.1 Tumor cell recognition

Activated macrophages that are able to recognize, bind and subsequently kill tumor cells, can distinguish between tumorigenic and non-tumorigenic cells, indicating that differences in cell membrane composition can be responsible for this specific tumor cell recognition. Several studies have demonstrated that the difference in the amount of phosphatidylserine in the outer membrane leaflet of tumor cells and non-tumorigenic cells is one of the factors responsible for specific tumor cell recognition (Utsugi et al, 1991; Elnemr et al, 2000). Upregulation of the amount of phosphatidylserine in the external part of the cell membrane by N-ethylmaleimide (Elnemr et al, 2000) or by addition of exogenous phosphatidylserine analogues leads to recognition and subsequent lysis or phagocytosis of tumor cells. In addition, carbohydrate structures present on tumor cells are also recognized by macrophages (Ichii et al 2000) indicating that altered glycosylation of cell surface molecules of tumor cells might be another mechanism for tumor cell recognition by macrophages. Some tumor antigens such as carcinoembryonic antigen (CEA) and Tn antigen (both human carcinoma-associated antigens) are carbohydrate structures. These carbohydrate moities bind to the lectin-like receptors present on macrophages (Suzuki et al, 1996).

Destruction of tumor cells in which macrophages are directly involved is thought to occur by two distinct mechanisms, i.e. (1) macrophage-mediated tumor cytotoxicity and (2) ADCC.

1.3.2 Macrophage-mediated tumor cytotoxicity

Macrophage-mediated tumor cytotoxicity is a slow, cell-to-cell contact-dependent process requiring 1–3 days. The susceptibility to macrophage-mediated tumor cytotoxicity varies greatly among different tumor cells (Daemen et al, 1989).
Heterogeneity in intercellular adhesion molecule-1 (ICAM-1) expression may underlie these differences. Involvement of cellular ICAM-1 in macrophage-mediated tumor cytotoxicity is confirmed by the following observations:

1. Decreased binding of macrophage-mediated tumor cytotoxicity-resistant tumor cells to monocytes coincided with lower levels of ICAM-1 (Jonjic et al., 1992);
2. Enhancement of ICAM-1 expression induced by cytokines resulted in increased vulnerability to tumoricidal macrophages, indicating that CD18/CD11-ICAM-1 adhesion between effector and target cells plays an important role in macrophage-mediated tumor cytotoxicity (Darcissac et al., 1996);
3. Anti-ICAM-1 or anti-CD18 antibodies caused substantial inhibition of tumoricidal activity of monocytes (Bemasconi et al., 1991). Besides differences in expression of adhesion molecules, also the cell cycle stage of the tumor cells may play a role in the susceptibility to lysis by tumoricidal monocytes. Horn et al., 1991 demonstrated that tumor cells leaving the cell cycle going into the quiescent state (G0) were no longer susceptible to lysis by monocytes. During coculture of monocytes with tumor cells, tumor cells can be driven into G0 phase by mediators released by activated monocytes. On the other hand, it was found that growth stimulatory signals such as epidermal growth factor, increased susceptibility to macrophage-mediated tumor cytotoxicity (Bosch et al., 1992).

1.3.3 Antibody-dependent cellular cytotoxicity

Both macrophages and monocytes are able to lyse tumor cells by ADCC. ADCC involves the recognition and binding of antibody-coated target cells via the Fc receptor on the effector cells (Kawase et al., 1985). In this process the Fc region of the cell-bound antibody provides an essential link between the effector and target cells necessary for intimate cell-to-cell contact and interaction. Upon occupancy and cross-linking of the Fc receptor, secretion of mediators involved in tumor cell killing occurs. The immunoglobulin classes IgG1, IgG2a, IgG2b, IgG3 have been demonstrated to be involved in ADCC (Kawase et al., 1985; Qi et al., 1995). The process of ADCC can be
completed either rapidly within a few hours or slowly, requiring 1–2 days depending on the activation state of the macrophage (responsive, primed or fully activated) and the antibody-isotype (Fan et al, 1991).

The mechanism by which target cells are killed during ADCC probably involves the generation of reactive oxygen species, including hydrogen peroxide and superoxide (Johnson et al, 1986). In addition, the release of TNF-α and IL-1, proteases and complement components may play a role in the lysis of the target cell. Munn and Cheung demonstrated that human macrophage-derived monocytes cultured with M-CSF were able to efficiently kill several types of antibody-coated tumor cells by phagocytosis. Phagocytosis was also observed in macrophage-mediated tumor cytotoxicity by (Gardner et al, 1991) who demonstrated by electron microscopy that tumor cells can be taken up by Kupffer cells.

In summary, tumor cells can be killed by macrophages either by macrophage-mediated tumor cytotoxicity or ADCC. In macrophage-mediated tumor cytotoxicity, adhesion molecules such as ICAM-1 play an important role in the interaction between tumor cells and macrophages, while in ADCC the presence of antibodies that recognize tumor antigens are needed. Both processes will end up in the release of cytotoxic mediators including TNF-α, IL-1, NO and reactive oxygen intermediates or phagocytosis.

1.4 Mediators in tumor cell killing

1.4.1 Tumor necrosis factor

The relative importance of mediators involved in tumor cell killing depends on the intrinsic susceptibility of different tumor cells as well as on the activation conditions of the effector cells. TNF, produced by activated macrophages, is known as a soluble protein (17 kDa) but also as a membrane-associated precursor molecule (26 kDa) (Jeong et al, 1997). After cleavage of the precursor molecule, the soluble form of TNF is secreted as a homotrimeric protein. Both the membrane-associated and the soluble molecules are effective in target cell killing (Marr et al, 1997). The mechanism by which
TNF is able to kill target cells involves the binding of TNF to TNF-receptors. Two distinct TNF receptors, which are present on the majority of cell types and tissues have been described, and are known as TNF receptors type 1 and 2 (TNF-R1, TNF-R2) (Hohmann et al, 1990). Probably, TNF-R2 is biologically less relevant than TNF-R1, as, in contrast to TNF-R1, binding of TNF to TNF-R2 is not sufficient to initiate cell killing. Binding to TNF-R1 can induce cytotoxicity, anti-viral activity, fibroblast proliferation, and induction of NF-κB. Binding to TNF-R2 induces thymocyte and cytotoxic T-lymphocyte proliferation (Tartaglia et al, 1993). After binding of TNF to its receptor, the receptor–TNF complex is internalized by receptor-mediated endocytosis, followed by the induction of multiple intracellular pathways. Among the effects reported are the generation of reactive oxygen intermediates in mitochondria preceding plasma membrane permeabilization, induction of iNOS-expression, DNA strand breaks and induction of serine proteolytic activity (Suffys et al, 1988). Ultimately, these processes can lead to cell death. (Sveinbjörnsson et al, 1997). Recently, also other members of the TNF family i.e. TNF-related apoptosis-inducing ligand (TRAIL) and Fas-ligand (FasL) have shown to be involved in cell death. Both ligands are expressed on the cell surface on monocytes or macrophages and induce apoptosis in cells expressing the receptor for TRAIL or FasL subsequent to the interaction between ligand and receptor (Griffith et al, 1999; Kiener et al, 1997). However, the role of these mediators in tumoricidal activity of monocytes or macrophages has to be elucidated.

1.4.2 Nitric oxide

NO produced by macrophages is synthesized enzymatically from arginine by nitric oxide synthase (NOS). NOS exists as a constitutive and as an inducible enzyme. Macrophages contain the inducible type of NOS, i.e. iNOS, which is found upon exposure to cytokines and bacterial cell wall products (Kroncke et al, 1995) under strict anaerobic conditions NO is stable but in the presence of oxygen NO is oxidized to higher nitrogen oxides, such as peroxynitrite (Xia). The end products are nitrite and nitrate. The toxic effects of NO and its derivatives on target cells are due to several mechanisms. First, NO mediates loss of iron from cells thereby inactivating iron–sulfur cluster-containing enzymes such as the citric acid cycle enzyme aconitase, NADH-ubiquinone
oxidoreductase and succinate-ubiquinone oxidoreductase of the mitochondrial electron transport (Henry et al, 1993). Second, NO is capable of inducing zinc release from zinc-containing proteins thereby inducing disulfide formation. This disulfide formation inhibits DNA-binding activity of zinc finger type transcription factors (Kroncke et al, 1994). Third, NO is capable of influencing the activity of ion channels thereby destroying the mitochondrial membrane potential (Richter et al, 1994).

Although the role of NO in the anti-tumor effect of rodent macrophages is well established (Takao et al, 1996). Its role in anti-tumor cytotoxicity in humans remains controversial. Several groups demonstrated NO secretion by human monocytes (Weinberg et al, 1995; Zembala et al, 1994). However, the conditions needed for NOS activation in human monocytes are different from those needed for the activation of rodent macrophages to secrete NO. Zembala et al. showed that human monocytes stimulated with various cytokines such as IFN-γ and TNF-α did not induce NO secretion, while under the same circumstances rodent peritoneal macrophages released large amounts of NO. However, upon coincubation of monocytes with some tumor cell types, NO secretion could be measured (Zembala et al, 1994). NO secretion or secretion of a NO metabolite in human cells was substantially lower than in rodent macrophages (Albina, 1995). The observation that human and rodent monocytes produce NO under different conditions suggests the occurrence of different pathways.

Taken together, TNF and NO are considered as the most important mediators directly involved in tumor cell killing. These mediators are most effective when there is close cell-to-cell contact. However, the observation that NO does not play such a prominent role in tumor cytotoxicity of human macrophages as is found for rodent macrophages, may indicate that also other (yet unknown) mediators must be capable of direct cell killing. Candidates are TNF-related ligands such as TRAIL and FasL.

1.5 Tumor-associated macrophages

1.5.1 Recruitment of tumor-associated macrophages

Although it has been established that macrophages are able to infiltrate tumors, the role of tumor-associated macrophages in tumor growth is still unclear. Infiltration of
macrophages in a tumor varies greatly from 0 to more than 80% of the total cellular tumor mass in animal models (Claasen et al, 1992). In general, the percentage of macrophages in human tumors is somewhat lower than in animals, ranging from 0 to 70%, depending on the tumor type (Merogi et al, 1997). There seems to be no or only very little correlation between immunogenicity of the tumor and the number of tumor-associated macrophages in a tumor (Zhang et al, 1997). Tumors sometimes produce factors that are likely to be responsible for accumulation of macrophages in the tumors. A cytokine with a molecular mass of 12 kDa referred to as monocyte chemotactic protein-1 (MCP-1), also known as monocyte chemotactic and activating factor, has been described to play an important role in attracting macrophages to tumor sites. Recruitment of peritumoral macrophages was reported to be independent of MCP-1 production; therefore MCP-1 seems responsible for recruitment of intratumoral macrophages only. Additional evidence for the involvement of MCP-1 in the recruitment of intratumoral macrophages was obtained in a study on the transfer of the MCP-1 gene into murine melanoma cells. Subcutaneous and intramuscular tumors induced by these transfected cells contained a significantly higher percentage of tumor-associated macrophages than tumors of untransfected cells. MCP-1 producing cells grew slower than untransfected cells in mice, resulting in a longer survival time. On the other hand, transfection with MCP-1 caused a modest but significant increase in tumorigenicity and tumor take rate and resulted in a decreased susceptibility to local IL-2 treatment. Similarly, Zhang et al. reported that human carcinoma cell lines producing high levels of MCP-1 showed earlier recruitment of tumor-associated macrophages while these cells had a lower tumor take rate than low- or non-producers. In vivo administration of antibodies to MCP-1 inhibited macrophage infiltration and stimulated tumor growth. This group also demonstrated that tumor growth was delayed or even abolished if at the time of subcutaneous inoculation of tumor cells (not producing MCP-1), the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine, was given. Since this peptide has been described to induce early monocyte recruitment into tumors these results suggest that at least in this model tumor-associated macrophages play a major role in tumor rejection. In addition, the number of intratumoral macrophages negatively correlated with tumor size (Zhang et al, 1997).
In several murine tumor models, however, no correlation was found between the number of tumor-associated macrophages and tumor growth rate or metastatic spread (Milas et al, 1987). In addition to MCP-1, also other chemoattractants such as MCP-2, MCP-3, the colony stimulating factors M-CSF and GM-CSF and vascular endothelial cell growth factor have been reported to be produced by some tumors (Scholl et al, 1994).

1.5.2 Tumor-associated macrophages and angiogenesis

The release by tumor cells of tumor-derived chemotactic factors capable of attracting monocytes to the tumor, suggests a favorable effect of the presence of macrophages for the tumor. This favorable effect may be explained by the fact that macrophages are involved in many processes; angiogenesis is one of them. Several studies reported a significant positive correlation among high vascularization of the tumor, increased macrophage infiltration and reduced overall survival in patients with breast carcinoma, pulmonary adenocarcinoma and endometrial carcinoma (Salvesen et al, 1999).

The spread and growth of tumor cells is dependent on the formation of new blood vessels and thus on angiogenesis. For this process the basement membrane and extracellular matrix have to be destroyed, followed by recruitment and/or proliferation of endothelial cells. Macrophages are able to promote these processes by

(1) The secretion of proteolytic enzymes that degrade extracellular matrix (Hildenbrand et al, 1998).

(2) The release of growth factors, among which are tumor growth factor-β (TGF-β), platelet-derived growth factor, vascular endothelial growth factor (VEGF), and TNF-α. The release of these factors plays a key role in angiogenesis. In vitro it has been demonstrated that production of the potent angiogenic factors IL-8 and VEGF from melanoma cells is upregulated through TNF-α and IL-1α secreted by activated macrophages (Torisu et al, 2000). Hence, macrophages can also indirectly induce the release of angiogenic factors by tumor cells themselves.

Among the enzymes responsible for destroying the basement membrane and extracellular matrix are matrix metalloproteinase-2 (MMP-2, gelatinase A, type IV collagenase) and MMP-9 (gelatinase B, type IV collagenase). The degradation of the basement membrane
and extracellular matrix provides a microenvironment in which activated endothelial cells can proliferate and migrate to form new blood vessels. Increased expression of MMP-2 and MMP-9 was found in several tumor types. Expression of MMP-2 and MMP-9 by host stromal cells, including macrophages, correlated with the depth of tumor invasion and histological grade in endometrial carcinoma (Iurlaro et al, 1999).

The angiogenic effects of most substances such as basic fibroblast growth factor, TGF-β, IL-8 and VEGF are mainly due to stimulation of migration and mitosis of endothelial cells. IL-8, a chemoattractant cytokine produced by several cell types among which are macrophages and tumor cells, has been shown to attract and activate neutrophils in inflammatory regions and to be angiogenic. IL-8 expression and survival is inversely correlated in nude mice inoculated with human ovarian carcinoma and also in patients with cervical cancers. VEGF also stimulates macrophage migration and is upregulated in macrophages by hypoxia (Fujimoto et al, 2000). In addition, it increases the vascular permeability and induces the production of plasminogen activator.

On the other hand, tumor-associated macrophages are able to directly or indirectly release substances, such as interferons, angiostatin, platelet factor-4 and thrombospondins that mediate inhibition of tumor and/or their metastases. These Factors inhibit angiogenesis by inhibiting endothelial cell proliferation. Angiostatin is formed by cleavage of plasminogen by metalloelastase. As metalloelastase is produced by (tumor-associated) macrophages, these macrophages seem to be responsible for the production of angiostatin. The angiostatin-induced inhibition of cell proliferation is due to an increase in apoptotic cells (Claesson-Welsh et al, 1998).

The role of tumor-associated macrophages in tumor angiogenesis also seems to be influenced by the stage of the tumor growth. In the early stages, the presence of tumor-associated macrophages will delay tumor cell proliferation. When the tumor grows beyond a size of 1 cm³, angiogenesis is thought to be necessary to provide the tumor with sufficient blood supply (Dvorak et al, 1986). At that time, tumor-associated macrophages may support tumor growth by secretion of angiogenic factors. In addition, the tumor may then be able to produce substances, such as prostaglandin E₂, that prevent macrophages from being tumor cytotoxic. Thus, early recruitment of cytotoxic macrophages may prevent outgrowth of tumor cells in cancer patients.
Summarized, whether tumor-associated macrophages will secrete tumor cytotoxic mediators or tumor growth supporting, i.e. proangiogenic, mediators probably depends on:

(1) The activation state of the macrophages, as is described earlier in this report; inhibition of tumor growth due to the presence of macrophages, is mostly observed in models in which early after tumor cell inoculation macrophages are recruited (Ushio et al, 1996).

(2) The tumor stage; stimulation of tumor development by tumor-associated macrophages is in general observed in established tumors containing immunosuppressive tumor-associated macrophages, or tumor-associated macrophages that become immunosuppressive during tumor development (Thomas et al, 1995).

(3) Microenvironmental influences, such as macrophage suppressive agents released by tumor cells, e.g. TGF-β and prostaglandin E₂ (Kambayashi et al, 1995) that are known to inhibit various functions of macrophages.

So, the onset of angiogenesis in tumors is determined by the local balance between pro-angiogenic and anti-angiogenic substances released by tumor-associated macrophages and tumors. The activation state of the macrophages, the tumor stage and microenvironmental conditions influence this balance.

1.6 Antigen presenting cells

Dendritic cells form a cell population strongly related to the macrophages and are ‘professional’ antigen-presenting cells. The primary function of dendritic cells is uptake and processing of antigens followed by the presentation of peptides on MHC I or MHC II molecules thereby initiating a T-lymphocyte response. Very low dendritic cell-to-T-cell ratios (1:50–1:200) suffice to provoke T-cell stimulation. Normally, the amounts of specific antigen–MHC complexes on tumor cells are small and the occurrence of being recognized by a T-cell clone is rare (Porgado et al, 1995; Bender et al, 1995; Banchereau et al, 1998). Dendritic cells provide a means to increase the contact probability between T-cells and antigens by capturing (tumor) antigens, process them into peptides and
display these in the context of MHC-molecules in the presence of costimulatory molecules.

The expression of surface antigens and the ability to take up and process antigens depends on the maturation stage of the dendritic cell. Immature dendritic cells are located in the peripheral tissues where they efficiently capture and process antigens. Antigen uptake is most efficiently accomplished via mannose-receptor-mediated endocytosis and macropinocytosis for taking up receptor-bound particles or large volumes of fluid, respectively. The mannose receptor has a broad specificity for sugars as well as hydrophobic molecules and therefore has a broad ligand specificity. The mannose receptor has the capacity to release the antigen at low pH in intracellular compartments and then to recycle to the cell surface. Thus it can deliver amounts of ligand that far exceed the number of receptors. Macropinocytosis is a constitutive process in dendritic cells and involves a cytoskeleton-dependent vacuole formation by which large vesicles (up to 3 μm) can be phagocytosed. Dendritic cells are able to retain intact proteins for up to 2 days before presenting them probably by the presence of retention compartments which are mildly acidic macropinosomes containing high concentrations of MHCII molecules (Winzler et al., 1997; Pierre et al., 1997).

Antigen uptake, but also cytokines involved in inflammation such as TNF and IL-1 and bacterial products, induce maturation and migration of dendritic cells from the periphery to lymph nodes where they present processed antigens to T-lymphocytes. Maturation of the dendritic cells may lead to further acidification of the macropinosomes which is needed for degradation and processing of the antigens. Maturation of dendritic cells is furthermore characterized by the disappearance of intracellular MHCII-rich compartments due to transportation of MHCII molecules to the cell surface. In addition, the level of macropinocytosis and endocytosis is reduced and expression of MHCII, costimulatory and adhesion molecules such as CD40, B7.2, and ICAM-1 (Winzler et al., 1997) is increased. This process of maturation is consistent with loss of the capacity of antigen uptake and processing, while on the other hand, the ability of antigen presentation is strongly increased.

Dendritic cells can be generated in large numbers either from CD34+ bone marrow cells or from peripheral blood monocytes when cultured in vitro in the presence of GM-CSF.
and TNF or IL-4. GM-CSF is necessary to stimulate proliferation of the precursor dendritic cells; IL-4 suppresses the differentiation into monocytes/macrophages, while TNF enhances maturation of dendritic cells (Lardon et al, 1997).

The possibility to culture large numbers of dendritic cells in vitro makes this cell type accessible for immunotherapeutic treatment of tumors. In animal studies, bone marrow derived dendritic cells pulsed with tumor or viral antigens were capable of inducing antigen specific cytotoxic T-cell responses in vivo. In addition, treatment with dendritic cells pulsed with well-defined tumor antigens as well as unfractionated acid-eluted tumor peptide mixtures protected mice against tumor challenge or suppressed tumor growth (Celluzzi et al, 1996).

Also human studies have been described in which autologous dendritic cells were pulsed with tumor specific peptides. In a clinical trial reported by (Hsu et al, 1996), four patients with B-cell lymphoma were treated with autologous dendritic cells derived from peripheral blood monocytes. The patients received two immunizations with dendritic cells pulsed with tumor specific peptide i.v. and two immunizations s.c. with soluble tumor protein to boost the primary response induced by peptide-loaded dendritic cells. In all four patients anti-tumor responses were observed and three patients showed clinical responses.

Several phase I clinical studies have been reported in which melanoma patients were intravenously, subcutaneously or intratumorally injected with dendritic cells pulsed with tumor specific peptides. The dendritic cells were derived from peripheral blood monocytes or hematopoietic progenitor cells. In these studies, immunotherapeutic treatment with dendritic cells was well tolerated. Complete regression of tumors was observed in 2 of 14, 1 of 16, 4 of 7 and 6 of 11 patients and stable disease was observed in 6 of 14 and 2 of 16 patients (Lau et al, 2001).

A phase II clinical study is described by (Lodge et al, 2000). In this study, 107 prostate cancer patients received six infusions of dendritic cells pulsed with peptides derived from prostate-specific membrane antigens every 6 weeks. Partial and complete responses were observed in 12 and 2 patients, respectively. The clinical responses tended to correlate with immunocompetence of the patients, i.e. a positive DTH response and high IFN-γ production by T-cells maintained throughout treatment.
Although dendritic cells are considered as the most important cell type in the generation of cytotoxic T-cells, also macrophages are able to stimulate the generation of cytotoxic T-cells by means of efficient antigen presentation. This means that macrophages have not only a non-specific, direct way of tumor cell killing as described earlier, but also a more specific and indirect way. An in vitro study of (Toujas et al, 1997) in which macrophages are directly compared with dendritic cells in cytotoxic T cell generation, showed that dendritic cells and macrophages were comparably effective in the activation of anti-melanoma cytotoxic T cell clones, after exogenous loading of melanoma specific peptides. (Apostolopoulos et al, 2000) immunized macrophages and dendritic cells ex vivo using oxidized mannan linked to MUC1 to target the mannose receptor and the MHC Class I antigen-presenting route. They demonstrated that murine mannose receptor bearing macrophages derived from peritoneal exudate cells that were cultured ex vivo with oxidized mannan linked to MUC1 efficiently present MUC1 to T cells leading to the generation of CTLs and protection from tumor challenge.

The results described suggest that immunization strategies using in vitro cultured antigen presenting cells pulsed with tumor antigens may be useful in inducing tumor-specific cytotoxic T-cell responses in cancer patients. Several clinical studies are ongoing and can be expected in the near future.

1.7 Macrophage activation in immunotherapy of human cancers

Macrophages/monocytes as a means to eliminate tumor cells. Two strategies can be distinguished:

(1) The in vivo activation of macrophages using biological response modifiers, such as MTP-PE, GM-CSF, M-CSF or IFN-

(2) Adoptive cellular immunotherapy, in which effector cells are isolated from the body and are re-infused several days later after in vitro activation of these cells with biological response modifiers.

1.7.1 Immunotherapy with biological response modifiers

In a MTP-PE clinical phase II study, relapsed osteosarcoma patients with pulmonary metastases were treated with liposomal MTP-PE. After i.v. infusion of the MTP-PE
liposomes, liposomes were found in the liver, spleen, lung and in and around lung metastases. While cytokine expression in monocytes was elevated, the expression of cytokines in lymphocytes was not influenced. Sixteen patients receiving MTP-PE liposomes for 24 weeks showed a prolongation in time to relapse (9.0 months) compared to that of a historical group (4.5 months). Liposomal MTP-PE treatment was well tolerated, as only mild side effects limited to fever, chills, myalgias and mild fatigue were observed (Asano et al, 1993; Kleinerman et al, 1992).

In a GM-CSF phase I/II study (Nagler et al, 1996), 34 patients with Hodgkin's lymphoma, non-Hodgkin's lymphoma, breast cancer or neuroblastoma received GM-CSF after autologous bone marrow transplantation. Cytotoxicity, ADCC, expression of the activation antigen CD16, and cytokine production were measured before, during and after GM-CSF administration. Within the group receiving GM-CSF, ADCC was higher during GM-CSF therapy than in the periods before and after GM-CSF administration. In addition, in vivo GM-CSF administration led to elevated levels of endogenous TNFα- and GM-CSF. Also clinical phase I and II studies have been conducted in which GM-CSF treatment was combined with other cytokines (such as IL-12 and IL-2) or monoclonal antibodies (Ragnhammar et al, 1996). In most studies an enhancement of ADCC was reported. Although stable disease was occasionally reported in these studies, explicit tumor regression upon GM-CSF administration has not been reported thus far.

1.7.2 Adoptive cellular immunotherapy

The development of leukopheresis and counterflow centrifugal elutriation has largely overcome the technical barrier of obtaining large numbers of monocytes needed for adoptive transfer in humans. To further improve the yield of isolated monocytes, patients have occasionally been pretreated with growth factors to increase the number of circulating blood monocytes. After isolation of the monocytes, cells were cultured for approximately 7 days to mature into macrophages. After 6 days of culture, biological response modifiers such as IFN-γ or sometimes MTP-PE or LPS are added to activate the macrophages. The subsequent re-infusion of the autologous monocytes into the host has been performed locoregionally as well as systemically. Generally, re-infusion of up to $2.7 \times 10^9$ (maximal number reported) of the autologous activated monocytes was well
tolerated. Low-grade fever was the most common side effect. Other side effects, which were usually mild, include malaise, and fatigue in case of i.v. and i.p. administration. In addition, a few patients receiving monocytes i.p., also indicated slight peritoneal irritation (Hennemann et al, 1998).

In only three cases in which patients received activated monocytes systemically, stable disease was reported. Also in three patients who received activated monocytes i.p., stable disease was observed (Eymard et al, 1996). Furthermore, in two patients ascites production stopped for approximately 3 months and a normalization of the elevated level of the serum tumor marker carcinoembryonic antigen in ascites was observed in one of these patients.

Upon i.p. administration of activated monocytes, the effector cells probably remain in the peritoneal cavity (in case of peritoneal cancers also the site of the tumor) for at least 5–7 days, and do not accumulate in other organs, as shown with indium-111 ($^{111}$In) labeled monocytes (Faradji et al, 1991). Since locoregional transfer of effector cells is more effective than systemic transfer of cells, accumulation of the monocytes in the peritoneal cavity might be essential for the observed therapeutic effect.

Although clinical responses in terms of measurable regression of tumor growth were almost absent, biological responses, such as elevated serum neopterin levels, IL-6 appearance in sera and ascitic fluids, increase in granulocyte count in blood, increase of IL-1, IL-6 and TNF-α in peritoneal fluids were reported in several studies (Andreesen et al, 1990; Hennemann et al, 1995; Faradji et al, 1991). In two studies circulating fibrin monomers were detected after i.v. administration of monocytes, indicating the induction of the coagulation cascade by the macrophages.

Although in vitro monocyte derived macrophages are more potent in lysing tumor cells than freshly isolated monocytes, no significant regression of tumor has been observed thus far after adoptive immunotherapy. These results are not unexpected, since all clinical studies so far have been phase I or II studies, which focus particularly on the cytotoxicity of the treatments rather than on the therapeutic effect. Furthermore, the number of patients entered in phase I or II studies are rather small and most patients have large established tumors that negatively affects the prognosis. However, locoregional
administration of tumoricidal macrophages in patients with progressive tumor in the peritoneal cavity might have an inhibiting effect on tumor growth.

The observation that most immunotherapeutic approaches described above, showed no or only minor side effects encourages further efforts to investigate its therapeutic potential in the treatment of minimal residual diseases. In case of minimal residual disease immunotherapeutic treatment aimed at macrophage activation may be more promising as the number of macrophages compared to the number of tumor cells is much larger. Furthermore, the use of combinations of macrophage activating mediators (Han et al., 1999) might improve the results of immunotherapy based macrophage activation.

1.8 Gene therapy

In the last decade, tumor cells genetically modified with cytokines, adhesion or MHC molecules have been examined for their anti-tumor effects in animal studies. These genetic modifications of tumor cells allow the induction of an immune response or facilitate tumor cell recognition or tumor cell killing by activated host immune cells. (Dranoff et al., 1993) investigated the effect of vaccination with both live and irradiated tumor cells retrovirally transfected with ten different cytokines, growth factors and adhesion molecules in a murine B16 melanoma model. They showed that transfection with the GM-CSF gene resulted in the best anti-tumor effect. The effect was ascribed to a potent, specific and long-lasting anti-tumor response. These results are supported by other murine studies, which also demonstrated protection to tumor challenges upon vaccination with GM-CSF transduced tumor cells (Mahvi et al, 1996). Sanda et al., 1994 vaccinated rats with GM-CSF secreting prostate tumor cells several days after tumor inoculation. A significant increase in the number of rats remaining tumor-free were observed when compared to the non-treated group and the group treated with non-transduced tumor cells with soluble GM-CSF.

Dow et al, 1998 reported on the successful treatment of canine melanoma by in vivo transfection with the GM-CSF gene. The 26 dogs that entered into the study had a histologic confirmation of spontaneously developed melanoma. The dogs were treated every 2 weeks for at least 12 weeks with intratumoral and peritumoral injections with lipid-complexed plasmid DNA encoding GM-CSF and staphylococcal enterotoxin B as a
superantigen. The therapy was well tolerated and 46% of the dogs showed complete or partial remission (best responses in animals with small tumors). Histological examination of the tumors showed that there was a strong increase in the number of infiltrated T-lymphocytes and macrophages compared to tumors injected with liposome-complexed empty DNA plasmid. In addition, there was a strong correlation between tumor regression and high levels of CTL response detected in peripheral blood lymphocytes.

As GM-CSF is known to stimulate macrophages and dendritic cells to enhance antigen presentation to T-cells, GM-CSF secretion is probably involved in the initiation of CTL induction. In most of the studies using tumor cells transfected with the GM-CSF gene, a strong increase in the number of antigen presenting cells (macrophages and/or dendritic cells), and CD4+ and CD8+ T-cells in the tumor were found (Soiffer et al., 1998;). Observation strongly suggests the involvement of these cells in the induction of systemic immune responses and tumor regression. Another mechanism of suppression of tumor metastases by GM-CSF secreting tumor cells has been described by Dong et al., 1998. They inoculated syngeneic mice and nude mice with B16-F10 or K-1735 melanoma cells engineered to produce high amounts of GM-CSF (>1 ng/10^6 cells) s.c. and observed slow-growing tumors in these animals. In addition, these tumor cells suppressed lung metastases of Lewis lung carcinoma, UV-2237 fibrosarcoma, K1735 and B16-F10 tumor cells not producing GM-CSF. The production of GM-CSF directly correlated with infiltrating macrophages and with their metalloelastase activity. As discussed above, metalloelastase can cleave plasminogen into angiostatin that is a potent inhibitor of angiogenesis by inhibiting the proliferation of endothelial cells. Thus, GM-CSF produced by tumor cells can upregulate metalloelastase in tumor-associated macrophages which leads to production of angiostatin and, hence, growth suppression of tumors and distant metastases.

Although most studies describe gene transfection with the GM-CSF gene, also other genes affecting the macrophage population have been used in experimental cancer gene therapy. Inoculation of mice with Lewis lung carcinoma cells retrovirally transfected with M-CSF resulted in an enhanced survival of the animals and prevention of lung metastases (Dong et al., 1999) Increasing the amount of M-CSF secreted, increased the survival rate. Furthermore, both vaccinations with M-CSF transfected
Lewis lung carcinoma cells before and after inoculation of the parental cells, significantly prolonged the survival of the mice. Similar results were found by Kimura et al, 1996, who observed prolonged survival in mice inoculated with M-CSF transfected L1210 mouse lymphoid cell line.

Dong et al, 1999 reported on a study in which highly metastatic PC-3M human prostate cancer cells transfected with the IFN-β gene were injected s.c. or into the prostate of nude mice. While rapidly growing tumors and regional lymph node metastases were observed in mice injected with the parental cells, no tumor growth and metastases were found in mice injected with IFN-β producing PC-3M cells. IFN-β secreting tumor cells were also able to suppress tumor growth of bystander non-transduced tumor cells. Immunohistochemical analysis revealed that IFN-β transfected PC-3M tumor cells were heavily infiltrated with macrophages in contrast to the parental tumor cells. In T and NK-cell compromised animals injected with IFN-β transfected PC-3M cells, only small avascularized tumors were present, in contrast to tumors of animals injected with parental PC-3M cells or cells transfected with a control vector. These results indicate that inhibition of vascularization and the infiltration of macrophages due to the production of IFN-β are responsible for tumor growth inhibition.

Besides animal studies, also human clinical phase I gene therapy studies have been described (Soiffer et al, 1998; Simons et al, 1997). Simons et al, 1997 immunized 18 patients suffering from renal cell carcinoma with irradiated autologous GM-CSF transfected renal cell carcinoma cells. To evaluate the cell-mediated immunity, a delayed-type-hypersensitivity assay was performed before and after treatment. The therapy was well tolerated, and one patient, who displayed the largest delayed-type-hypersensitivity response, showed an objective partial response (reduction of pulmonary metastases). Injection sites i.d. and s.c. were strongly infiltrated with antigen presenting cells and T-lymphocytes.

Similar results were observed in a study with 21 melanoma patients who were treated with irradiated autologous melanoma cells transduced with the GM-CSF gene (Soiffer et al, 1998). Also high infiltration of antigen presenting cells and T-lymphocytes and extensive tumor cell destruction was observed upon vaccination with the GM-CSF
transfected tumor cells. One partial response, one mixed response and three minor responses were observed.

As clinical phase I studies aim at evaluation of safety and toxicity with increasing doses of treatment rather than therapeutic effects, these studies are often performed in patients with advanced tumor disease not amendable to standard therapy. Therefore, as the prognosis in these patients is often bad and the number of patients small, clinical responses are generally rare in phase I studies.

Taken together, the results in animal as well as in human studies indicate that this kind of immune/gene therapy can enhance the host immune response towards tumor cells. Antigen presenting cells, consisting of macrophages and dendritic cells, together with T-lymphocytes appear to be involved in this process. Additional clinical studies will have to be conducted to elucidate the effectiveness of immune/gene therapy.