Chapter III

Generation of an effective cellular tumor vaccine by fusion of professional antigen presenting cells with fibrosarcoma cells

5.12 Induction of a polyvalent immune response enhances selective pressure and effectively protects mice from tumor growth

In tumor cells, abnormal protein expression results from DNA mutations or fusion associated with carcinogenesis or tumor progression. Those abnormal, often clearly defined proteins should be recognized by the immune system and induce an immune response leading to tumor regression. Actually, most tumors escape the immune response through a specific tolerance, because a defect in tumor-antigen presentation to the host immune system is involved (287-289). T cell activation involves the delivery of two independent signals to the naive T cell (290, 291). The first signal occurs with engagement of the TCR and the recognition of tumor associated antigen peptides complexed with MHC molecules. T cell activation requires also a costimulatory signal delivered to the CD28 receptor on T cells by the B7 family of molecules expressed by the antigen presenting cell. Most tumor cells express MHC class I molecules, a minority also express MHC class II molecules and only a few lymphoma have been reported to express B7 (292). For this reason most tumor cells are not able to efficiently present their specific antigens to competent T cells.
Among antigen presenting cells, dendritic cells, unlike B lymphocytes and macrophages, are the only cells able to stimulate both naive and memory T lymphocytes (293, 294). Actually, dendritic cells are supposed to take place in the anti-tumor immune response, and dendritic cell infiltrates inside numerous neoplasms are often associated to an immune response against tumors (295, 296). A combination of various tumor antigens with the antigen presenting properties of DCs might be of potential use in immunotherapy. The fusion of tumor cells with professional APCs generates a cellular vaccine where expression of tumor antigens and the simultaneous antigen presenting capacity of APCs can be exploited to induce specific anti-tumoral lymphocytes. Since tumors arise due to multiple genetic defects the recipient is vaccinated against many different tumor antigens. Immunization against only one single tumor antigen might lead to resistant cell variants from experimental tumors that have lost or dramatically downregulated the expression of the specific tumor-antigen. However, the use of a cellular vaccine combining features of both tumor cell and APC enlarges selective pressure on the tumor and is more promising. To study this strategy, DCs and the tumor cell line Meth A were stable transfected with a selection marker. Resistant clones were used for cell fusion. After double selection positive cell hybrid clones were analyzed for expression of MHC- and costimulatory molecules. Characterized clones were used for immunization of mice and after inoculation with primary tumor cells the immune response was evaluated.

5.13 Cell fusion of Meth A tumor cells with the dendritic cell line D2SC-1

The retroviral vector pBABEhygro was introduced into Meth A tumor cells by calcium phosphate precipitation to obtain hygromycin resistant clones. D2SC-1 cells were stably transfected by pBABEpuro. Cells were grown in RPMI, 10% FCS, 2mM L-glutamine, 5 µg/ml puromycin or 100 µg/ml hygromycin B (Boehringer Mannheim, Germany). Selective medium was replaced every third day. After 4 weeks resistant clones were pooled and used for cell fusion. D2SC-1 cells and Meth A tumor cells were co-pelleted by centrifugation and fused by addition of polyethylene glycol to the pellet. Dendritic cells and Meth A tumor cells were used in a ratio of 1:5. Cells were centrifuged and the PEG solution diluted by slow addition of medium. Fused cells were centrifuged, resuspended in puromycin/hygromycin

selection medium and aliquoted into 96-well microtiter plates. After 4 weeks 56 different cell hybrid clones were isolated from the microtiter plates and assayed for expression of major histocompatibility complexes class I or II and the co-stimulatory molecules B7-1 and B7-2 (see appendix). These molecules are essential for the induction of cellular and humoral immune responses (297, 298). The parental cell line D2SC-1 shows high expression of B7.1, B7.2, MHC class I and MHC class II molecules that are found on all professional APCs (Fig. 5.30). On the other hand, Meth A tumor cells only express MHC class I molecules that are necessary for antigen-recognition of cytotoxic T lymphocytes, whereas the costimulatory molecules B7-1 and B7-2 or MHC class II molecules were not detected on the cell surface (Fig. 5.30). The downregulation of costimulatory molecules on these tumor cells might serves as an immunological escape mechanism and is a general phenomenon observed in cancer.
However, most cell fusion hybrid clones show expression of all of these cell surface antigens even if the number of these molecules is varying from hybrid clone to hybrid clone, as illustrated by FACS analysis (Fig. 5.30). By combining effective antigen presenting properties of dendritic cells with antigenic properties from tumor cells, tumor antigens can now efficiently be presented to immune effector cells that closely expand and form potent and protective anti-tumor activity.

5.14 Immunization of Balb/c mice with cell hybrid clones

Tumor cells can escape immune surveillance because they do not express signals that are essential for activation of the host immune system (287-289). On the other hand, DCs are specialized for antigen uptake, processing, and presentation to T cells and play a central role in the induction of primary antigen-specific immune responses (293, 294). A fusion of a tumor cell with a dendritic cell produces a hybrid cell that both expresses tumor specific antigens and has the machinery for antigen presentation and T cell activation. We wanted to use the fusion cells as a cellular vaccine and examine their potential as an effective cancer immunotherapy approach.
Cell hybrid clones were obtained by fusion of Meth A tumor cells with D2SC-1 cells and maintained in selective medium in the presence of 5 μg/ml puromycin, 100 μg/ml hygromycin. 24 clones were randomly chosen for immunization of female, 6 to 8 weeks old Balb/c mice. 7 x 10^6 hybrid cells were injected subcutaneously into the flank of recipient mice. One week after inoculation with the cellular vaccine tumor nodules 2 to 3 mm in diameter were identified at the site of injection that were eliminated within the next two weeks (data not shown). However, five of these nodules developed to solid tumors and eventually killed the mice (Tab. 5.3). Four weeks after immunization the remaining mice were inoculated with the parental Meth A tumor cells and the formation of metastasis (after intravenous injection) or solid tumor growth (subcutaneous injection) was used to assess the efficacy of the cellular vaccine.
Table 5.3 summarizes the results of our experiments. Fusion of D2SC-1 cells with the tumor cell line Meth A produced hybrid cells that lost their tumorigenity and became immunogenic. Fusion hybrid cell lines protected mice from growth of the parental Meth A tumor cells. Although Meth A tumor cells formed small nodules at the injection site they were quickly eliminated within ten days. Mice were also protected from disseminated metastases as verified by histopathochemistry (data not shown). On the other hand, mice that did not obtain the cellular vaccine were not protected from Meth A tumor growth and formed lethal solid tumors or disseminated metastases in the lung.

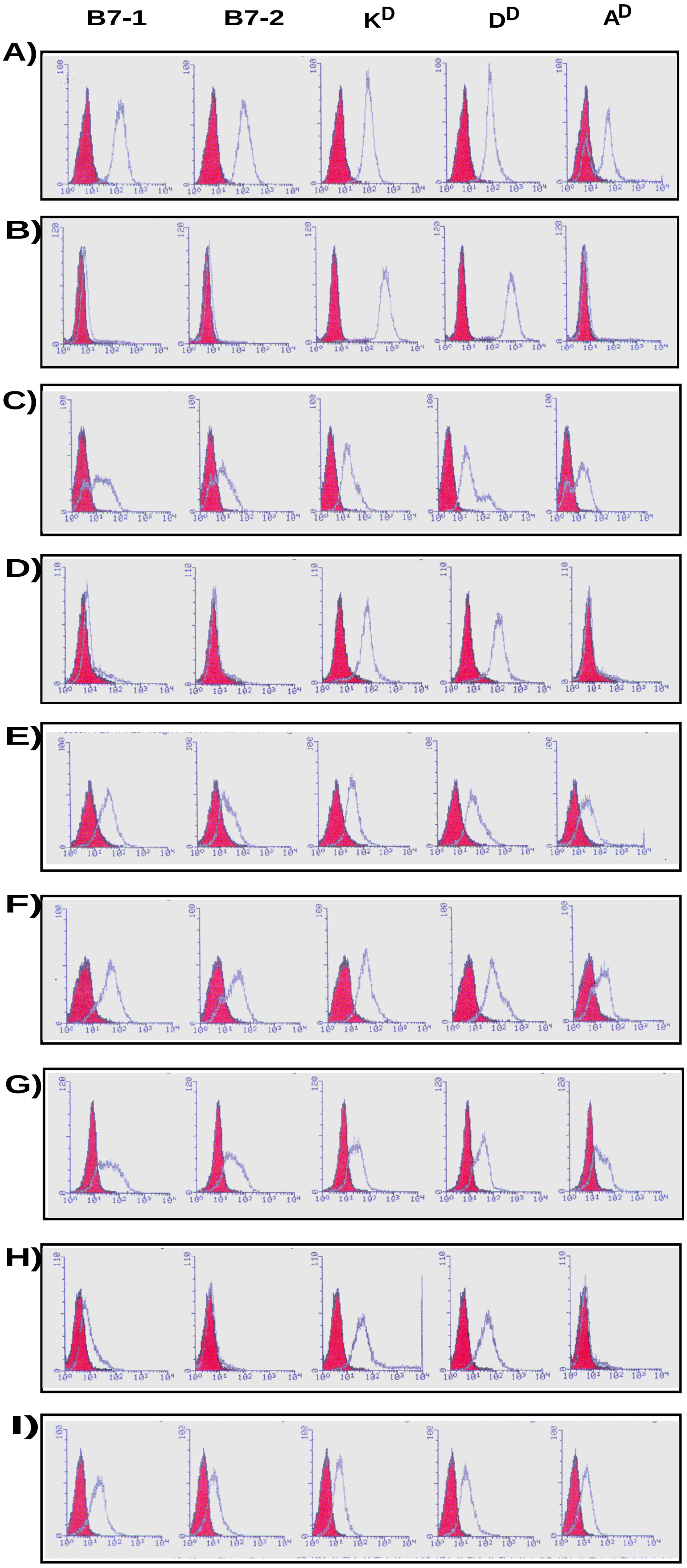

Fig. 5.30 Expression of MHC class I and class II antigens, B7.1 and B7.2 co-stimulatory molecules on the dendritic cell line D2SC-1 **(A)**, Meth-A tumor cells **(B)** and cell fusion hybride clones x40 **(C)**, x13 **(D)**, x6 **(E)**, x27 **(F)**, x39 **(G)**, O **(H)** and F **(I)**. Cells were washed with phosphate buffered saline and stained with FITC-labelled monoclonal antibodies to mouse B7.1 (16-10A1), mouse B7.2 (GL1), K^D (SF1-1.1), D^D (34-2-12) and PE-labelled antiboidies to mouse A^D (AMS-32.1) for 30 min. on ice.
Samples were than washed and analyzed ir a FACScan (Becton Dickinson, Heidelberg, Germany). Solid areas are untreated cells, open areas are stained with specific antibodies.

Immunization with fusion hybrids protects mice from parental Meth A tumor growth

hybride clones	tumor formation of cell hybrides in Balb/c mice	tumor formation of cell hybrides in nude mice	rejection of primary tumor
x10	-	+	+
x17	-	+	+
x39	-	+	+
x9	-	+	+
x41	-	+	+
x27	-	+	+
x3	-	+	+
x16	+	+	o
x14	+	+	o
M	-	+	+
x38	+	+	o
x15	-	+	+
x11	-	+	+
x23	-	+	+
x13	+	+	o
x36	-	+	+
C	-	+	+
O	-	+	+
x2	-	+	-
x21	-	+	+
x40	-	+	+
H	-	+	+
x19	+	+	o
K	-	+	+

Tab. 5.3 Meth A tumor cells were fused with the dendritic cell line D2SC-1. Fusion hybrid clones were used for immunization of Balb/c mice or nude mice. All fusion hybrids formed tumors on nude mice whereas in Balb/c mice the hybrid clones lost tumorigenity (except the hybrid clones X16, X14, X38, X13, and X19 that formed lethal tumors). Recipient mice that obtained the cellular vaccine were resistant to the parental Meth A tumor cell line and rejected tumor cells when injected subcutaneously or intravenously. Clone X2 was not able to induce protective immunity to Meth A tumor cells.

5.15 Immune effector mechanisms responsible for Meth A tumor rejection

5.15.1 Immunization of mice with hybrid cell fusions induces B cell activation and recognition of tumor specific antigens in Balb/c mice

Dendritic cells have a major impact on the regulation of immune responses. They are known to influence B cell growth and immunoglobulin secretion (182-184). DCs activate and expand Th-cells, which in turn induce B cell growth and antibody production. By secretion of soluble factors including IL-6, IL-12, and TNF-α DCs directly stimulate growth and differentiation of B cells and the production of antibodies. Particularly follicular dendritic cells are believed to be important in humoral immunity because they sustain viability, growth and differentiation of activated B cells (299-301).

DCs fused to tumor cells most probably maintain these properties and therefore hybrid fusion cells were analyzed for their ability to induce an antibody-mediated immune response. Recipient Balb/c mice were immunized with cell hybrid clones and inoculated with Meth A tumor cells. 10 days after tumor cell inoculation blood samples were taken from the lateral tail vein. The serum was analyzed for the presence of Meth A specific antibodies. Meth A specific antibodies most probably belong to the IgG or IgM isotype, because after first antigenic encounter mature B cells are known to express IgM and then increasing amounts of the IgG isotype with growing affinity due to a process of isotype switching and affinity maturation. Results are illustrated in Fig. 5.31. Mice that rejected the parental tumor after vaccination displayed Meth A specific antibodies. These antibodies were not present in the serum of untreated mice. Also mice that were inoculated only with tumor cells or with cell clones that did not protect from Meth A tumor growth did not display antibodies that bound to proteins from Meth A tumor cells.

These experiments substantially support the idea that Meth A specific antibodies are induced only after immunization with cell fusion hybrids and exposure to tumor cells, and that they are not present in the non-vaccinated recipient. A specific antibody-profile is only displayed in tumor resistant mice and therefore we speculate that these antibodies might be important for the process of tumor rejection. In future experiments we want to identify the tumor-antigens recognized by these antibodies by immunoprecipitation and microsequencing in cooperation with the Max Plank Institute of Immunology, Freiburg, Germany.

Immunization of mice with fusion hybrids induces Meth A specific B lymphocytes

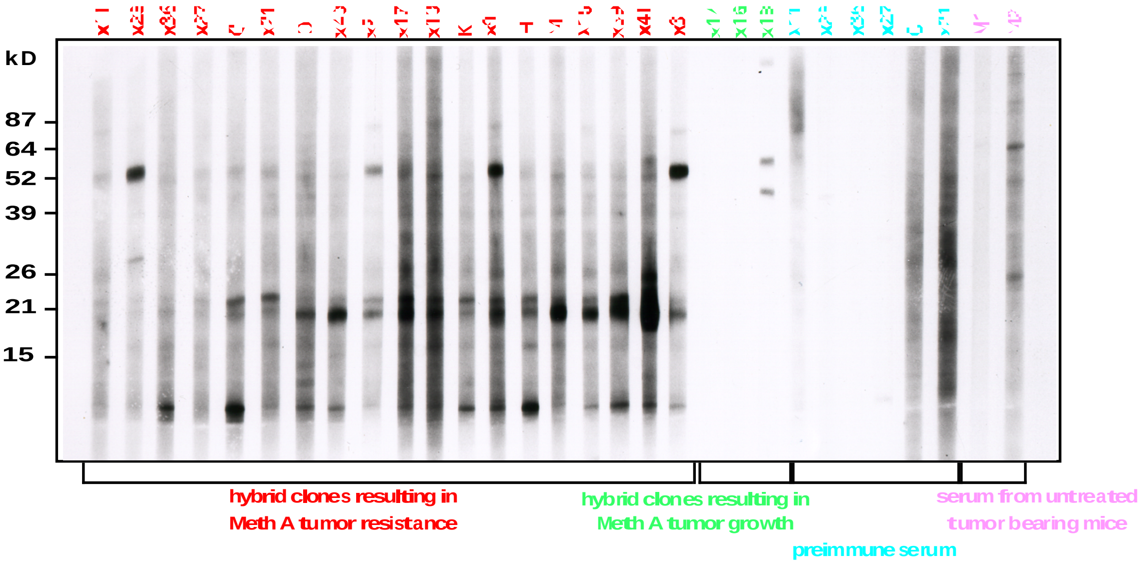

Fig. 5.31 Immunization of Balb/c mice with fusion hybrid cells induces the formation of Meth A specific antibodies. Recipient Balb/c mice were immunized with 7 x 106 fusion hybrid cells and inoculated with 5 x 106 Meth A tumor cells one month after immunization. Blood sera were isolated 10 days post tumor cell inoculation. To identify Meth A specific antibodies Meth A tumor cells were lysed, separated by SDS-PAGE and used in a Western blot experiment. Mouse sera were analyzed at a dilution of 1:400. The immunization of donor mice is indicated on the top of the blot. Meth A specific IgG antibodies, recognizing proteins of 53 kD, 21 kD, and 16 kD were exclusively identified in tumor resistant mice.

5.15.2 The activation of B cells alone is not sufficient for tumor rejection

Antibodies, secreted by B cells can bind to tumor cells and trigger specific effector functions or they are a consequence of the accessibility of tumor antigens due to tumor cell degradation. It is now widely accepted that the immunoglobulin-isotypes determine the effector functions of humoral immunity. In our experiments Meth A specific antibodies were detected by a secondary IgG specific, HRP labeled goat anti-mouse antibody (Fig. 5.31). IgG antibodies are closely connected to the complement system, consisting of a family of serum proteins that can be activated by a proteolytic cascade to generate effector molecules. Complement protein binds to the Fc region of antigen-antibody complexes and trigger the classical complement pathway. IgGs can also coat antigenic particles and the bound IgGs serve to enhance the efficiency of phagocytosis by mononuclear phagocytes and granulocytes that have the ability to ingest opsonized particles as a prelude to intracellular killing and degradation. IgGs also promote the antibody dependent cell mediated cytotoxicity. Different leukocyte populations including neutrophils, eosinophils, mononuclear phagocytes and NK cells are capable of lysing various target cells when precoated with specific IgGs. Recognition of aggregated antibodies occurs through low affinity receptors for Fcγ, called the CD16 receptor. Lysis of target cells is performed by secretion of cytokines such as TNF or IFN-γ and release of granules.

However, in the next experiment we show that the presence of B cells alone is not sufficient for the rejection of tumors. Female, athymic nude mice were immunized with cell fusion hybrid clones by subcutaneous injection into the flank of recipient animals. In contrast to immunocompetent Balb/c mice cell fusion hybrids formed aggressive tumors in the absence of T lymphocytes (Fig. 5.32). From this model system we suggest two requirements for an effective anti-tumor response. The tumorigenity of Meth A tumor cells is not lost by fusion to dendritic cells in the absence of T lymphocytes. This might be due to the fact that the presence of Th cells is essential for B cell activation and the induction of a humoral immune response. Helper T cells deliver contact-mediated signals to B cells and secrete cytokines that induce growth and differentiation of B cells and influence the nature and magnitude of the antibody response. Non-stimulated B cells require contact with helper T cells whereas recently stimulated B cells may be fully responsive to cytokines alone. However, B cells might not be the major effector cells participating in tumor rejection. The recognition of tumor cells by $CD8^+$ T lymphocytes depends on the endogenous processing of tumor antigens and their presentation via MHC class I complexes. Most nuclear cells express MHC class I molecules and can therefore present tumor antigens but only APCs are coated with costimulatory molecules that are necessary for T cell activation. By fusion of Meth A tumor cells with DCs the resulting fusion hybrid cell combines both the tumor-antigenes and the machinery that is necessary for efficient T cell activation. When individuals are immunized with the cellular vaccine cytotoxic T lymphocytes might be activated via the biological pathways and induce a potent anti-tumor response.

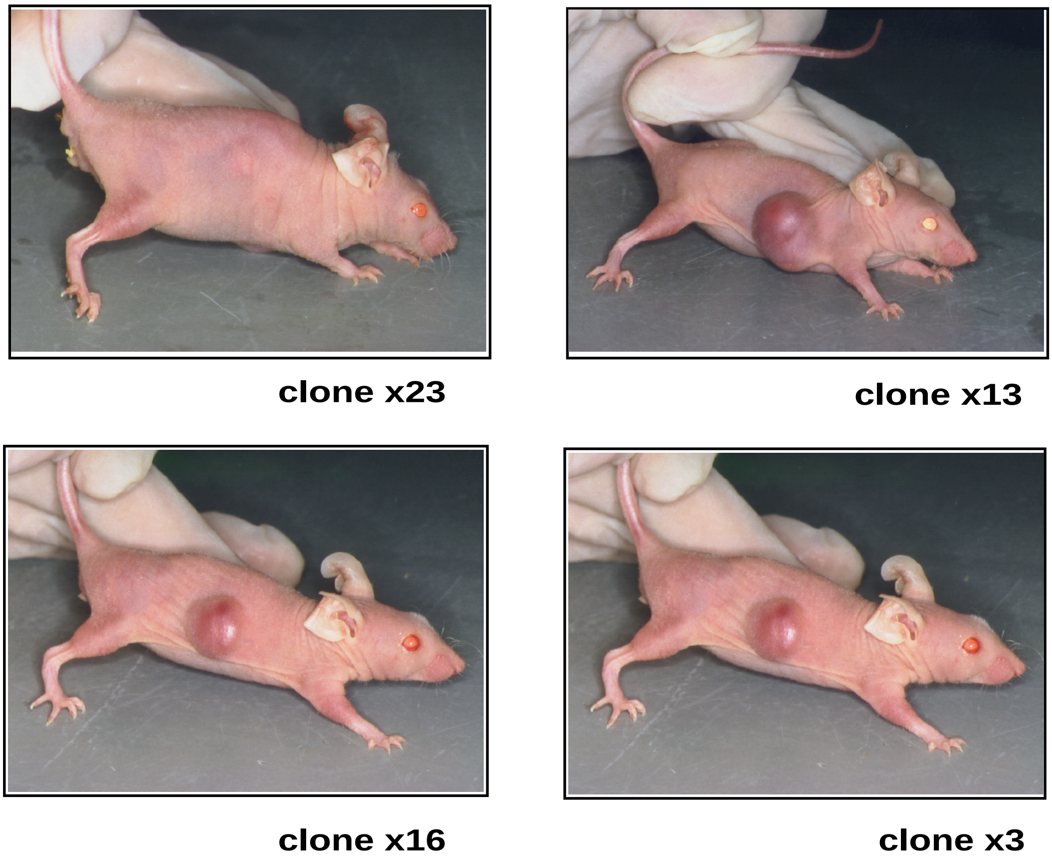

Fig. 5.32 Hybrid fusion cell clones stay tumorigenic in athymic, nude mice. Nude mice were subcutaneously inoculated with 7 x 10^6 fusion hybrid cells. Growth of cells was measured every third day with a caliper rule. All hybrid clones formed lethal solid tumors, however, kinetics of tumor growth differed substantially depending on the cell fusion clone. Tumors of cell hybrids are illustrated at day 23 post injection. Clones are indicated on the bottom of each blot.

5.15.3 T lymphocytes are part of the mechanism for Meth A tumor rejection

Growth of hybrid clones on nude mice indicated an active participation of T lymphocytes in tumor rejection. These mice lack the T lymphocyte dependent immune responses in contrast to fully immunocompetent Balb/c mice that rejected the same cell hybrid clones. Indeed we could demonstrate a specific induction of T lymphocytes by a ^{3}H-proliferation assay. Tumor resistant, fusion hybrid clone vaccinated animals were sacrificed to isolate spleenic T lymphocytes. T cells were plated in round bottom microtiter plates at 10^6 cells per well or titrated 1:4 in triplicates. Proliferation was induced by coculture of 2 x 10^4 irradiated hybrid target cells (200 grey). Cells were cultured for 72 hrs. in 200 µl DMEM, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 0.5 mM β-mercaptoethanol, and 0.4 ng/ml IL-2. 1 µCi of (^{3}H)-thymidine was added for the last 15 hrs.. The labeled cells were harvested on cellulose filters and analyzed in a liquid scintillation β-counter. T cells from untreated Balb/c mice injected only with primary Meth A cells served as a negative control. Our results clearly demonstrate the presence of fusion hybrid specific T cells in vaccinated animals, whereas untreated controls did not display this phenotype (Fig. 5.33).

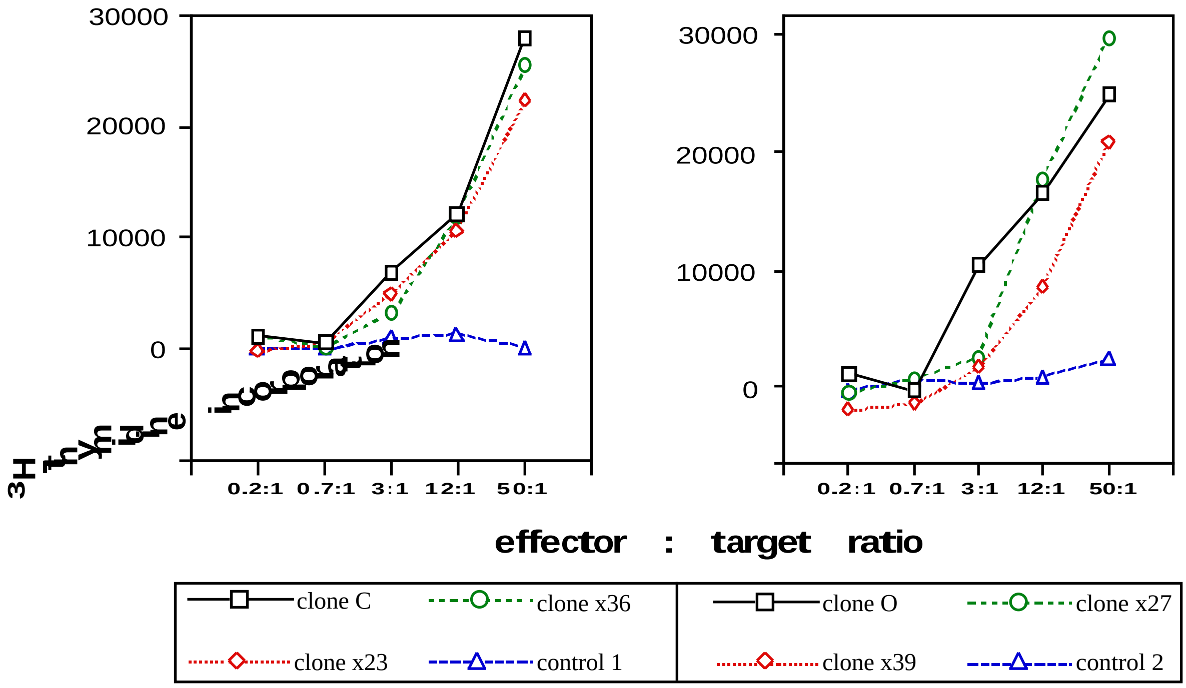

Fig. 5.33 (^{3}H)-proliferation assay and detection of fusion hybrid reactive T lymphocytes. Fusion hybrid specific T cells were demonstrated in immunized Balb/c mice treated with the cellular vaccine. Mice were injected with 7 x 10^6 fusion hybrid cells (clone O, C, x23, x27, x36 or x39). Lymphocytes from untreated mice, injected only with Meth A tumor cells 10 days before sectomy served as a negative control. 2 x 10^4 fusion hybrid target cells were seeded per well and mixed with varying effector cell ratio (50:1 to 1:5).

Due to the fact that nude mice were not protected from lethal tumor growth and only immunized immunocompetent Balb/c mice displayed hybrid cell responsive T cells we think that the presence of T lymphocytes is a necessary requirement for tumor cell rejection in healthy individuals. In the next set of experiments we plan to determine whether tumor rejection is primary mediated by CD4+ or CD8+ T cells. *In vivo* depletion studies using anti-CD4 or anti-CD8 antibodies to eliminate single effector populations by specific complement activated lysis are ongoing.

VI. Discussion

Traditional cancer chemotherapy plays a major role in the treatment of hematological and solid tumors. Since 1955 over 700.000 compounds and extracts have been screened for anti-neoplastic properties in the States. In 1945 there was only one drug known to be effective, namely nitrogen mustard. Today, there are nearly 50 chemotherapeutic agents used, single or in combination, in the treatment of malignancies.
The development of cancer immunotherapy was characterized by a wave of excitement similar to the antibiotic treatment of infectious diseases. In 1943, after it had been observed that World War II soldiers exposed to nitrogen mustard gases had a reduction in the size of their lymph nodes, mechlorethamine hydrochloride was first used to treat Hodgkin's disease. A rapid decrease in the patients' lymph nodes was documented but remissions were very transient. At about the same time the antimetabolite antibiotic methotrexate was used to treat acute lymphocytic leukemia. In 1955 the use of methotrexate in gestational choriocarcinoma resulted in the first cancer cures.
The lack of cure in other cancer patients similarly treated prompted investigators to examine combinations of chemotherapeutic agents comparable to the treatment of infections where a combination of antibiotics is used. In the latter, up to 22 percent complete responses were recorded. However, substantial toxicity and drug-related deaths were major deterrents. In 1958 the first randomized study in children with acute lymphocytic leukemia was reported and remarkable responses were observed.
In 1964 Skipper and his colleagues began the first studies to evaluate drug combinations. They utilized the murine leukemia L1210 cell line to examine the growth characteristics of tumors in a mouse model and characterized the response of cancer cells to chemotherapy. Because of their success other researchers were also prompted to study cancer growth with the goal of improving the ability to destroy cancer cells.
Today, cancers are mainly cured by surgical resection. Chemotherapy for cancer is used primarily in the treatment of non-operable or metastatic malignancy or as an adjunct to primary surgical treatment. Success in the treatment of malignancies is based on the optimization of known therapeutic agents in concentration and combination. Although the primary goal of chemotherapy is to cure, most patients are, in fact, not cured. Nearly all patients have some tumor response in their therapy, but only a small number have their lives substantially prolonged. Therefore, to improve the ability to treat cancer, new cancer therapeutic approaches need to be developed.

6.1 A novel cancer therapeutic approach: immunotherapy

The therapy of human cancer is aimed to specifically kill the tumor cells while sparing normal cells. Surgical resection and chemotherapy are often not able to control the progression of a malignant disease. Alternative strategies are needed, especially for patients with metastatic or non-operable cancer. Recent progress in molecular biology has permitted us to better understand the mechanisms of carcinogenesis, tumor progression, and host antitumor immune response. These developments have laid the conceptual and technical foundation of alternative therapeutic strategies for cancer treatment. Three innovative therapeutic approaches that target cancer cells are presented by this work and will be discussed: pDNA immunization, transduction of antigen presenting cells with a tumor antigen, and fusion of dendritic cells with a tumor cell line. These approaches attempt to improve the antitumor response of the host by increasing the antitumor activity of the immunologic effector mechanisms infiltrating the tumor. Two of these therapeutic approaches were targeted at the p53 tumor antigen.

6.2 Different reasons to use p53 as a target molecule

6.2.1 Mutated p53 genes are a common phenomenon in human malignancies

The p53 tumor suppressor gene is, so far, the most commonly mutated gene identified in human malignancies. Mutations occur in more than 50% of human malignancies and in diverse tumor types (305). Genetic alterations have been implicated in a high percentage of colon cancer, ovarian cancer, bladder cancer, and B-cell acute lymphocytic leukemia (306). It was also reported that germ-line p53 mutations were associated with the Li-Fraumeni syndrome, a familiar cancer disease (307). Commonly the p53 locus is mutated by missense mutation in the region encoding the DNA binding domain (residues 102-290). In cancer it has been found that the major hot spots for mutation generally abrogate DNA-binding without affecting the conformation of the molecule (308).
In contrast to wild type p53, many mutants accumulate in cancer cells. This suggests that cancer cells select for p53 mutations and indeed many mutants have gained an oncogeneic phenotype. In cells containing both wild type and mutant alleles this phenotype could result from a dominant-negative inhibition of wild-type p53 by coexpressed mutant. However, a recent study shows that mutant p53 also enhances tumorigenicity of p53 deficient cells, implying that mutant p53 does not only act by negative dominance but exerts oncogeneic functions of its own (309). In nontransformed cells p53 has an extremely short life of 5 to 40 minutes and is nearly undetectable by immunohistochemistry (310, 311). However, the stability of p53 is markedly increased in various types of transformed cells and results in increased levels of p53. In a number of cases, this stability is correlated with the formation of a complex between p53 and other proteins, such as the simian virus SV40 large T-antigen (312), or the adenoviral 58 kD E1b protein (313), or cellular heat shock proteins (314, 315).

6.2.2 p53 is involved in drug resistance

Resistance of tumor cells to radiation and chemotherapy remains a significant obstacle in the treatment of cancer. Some tumors fail to respond to either form of treatment or become nonresponsive on tumor relapse. It is now apparent that many anti-cancer agents used in the current clinical setting induce tumor cell apoptosis, a genetically regulated form of cell death (316, 317). However, molecular events, such as mutations in apoptotic programs might occur after drug-targeted interaction and may produce a pleiotropic resistance to anticancer agents.
It has been observed that cancer therapy is less effective in patients harboring tumors with p53 mutations. The pattern and frequency of p53 mutations in various tumor types roughly correlate the tumor's responsiveness to therapy. Tumor types displaying a high frequency of p53 mutations are generally not as responsive as tumors that rarely harbor p53 mutations (318). Numerous studies associate p53 mutations with poor patients’ prognosis, hence a reduced probability that therapeutic intervention will be effective. Some examples include carcinomas of the breast, lung, prostate, and bladder, soft tissue sarcomas, Wilms' tumors and various leukemia and lymphomas (318-320). Cells acquiring p53 mutations more readily survive chemotherapy and predominate on tumor relapse. Relaps of cancer is is associated with p53 mutations and such patients are much less likely to enter a second remission compared with patients whose condition relapses with normal p53 (321-326). Generally these patients displayed shorter survival when compared to patients with normal p53 (325).
In certain solid tumors, p53 mutations are associated with reduced apoptosis in tumors that are often refractory to therapeutic intervention. For example, p53 mutations are tightly linked to reduced apoptosis in anaplastic Wilms' tumor, an

aggressive subtype that responds poorly to chemotherapy (327). Similarly, p53 mutations occur at more advanced stages of colon cancer, a period when tumors display dramatic decreases in apoptosis (328, 329). The role of p53 in therapy induced apoptosis can explain the selection for p53 mutations following cancer therapy. However, many tumors display p53 mutations at diagnosis. A recent study indicates that hypoxia can induce apoptosis in oncogenetically transformed cells by a p53 dependent mechanism (330). Cells acquiring p53 mutations would have a survival advantage in such an environment, leading to their clonal expansion. Because the same mutations that diminish hypoxia-induced apoptosis also promote resistance to radiation and chemotherapy, selective pressure to inactivate apoptosis during tumorigenesis may indirectly produce drug resistance prior therapeutic intervention.

The selection for mutant p53 positive tumor cells by chemotherapy makes p53 an ideal target for new therapeutic approaches that do not depend on the activation of p53 dependent apoptotic pathways. New approaches might attempt to improve the host antitumor response by increasing the antitumor activity of the immunologic effector mechanisms infiltrating the tumor. Three different approaches will be discussed in this work.

6.2.3 Mutated p53 can induce a humoral or a cellular immune response

Not all therapies that target p53 during cancer therapy require p53's role in apoptosis. Mutant p53 alleles encode an altered protein that could serve as an antigen to direct an immune response against the tumor (331). Lately, cell mediated immune responses against tumors are becoming a focus of cancer immunotherapy. Usually class I MHC restricted CD8+ CTLs are considered the critical effector cells involved in an antitumor immune response (332-334). The presence of mutated gene products makes malignant cells differ from normal cells and might serve as a specific antigen marker. Indeed, p53 is the most commonly mutated gene in human cancer and recent data predispose p53 as the optimal target gene for our experiments. Numerous studies have shown that p53 is a tumor specific antigen that can induce immune responses in cancer patients. A tumor-associated humoral immune response directed against p53 has been detected in cancer patients with breast cancer (91), B cell lymphoma (89), and lung cancer (90). The antibody production was connected to overexpression of the p53 gene in tumor cells. Mutant p53 can also induce cytolytic immune responses. Yanuck et al. demonstrated that processed peptides of p53 could bind to MHC class I molecules and induce cytolytic T lymphocytes (94). In these experiments spleen cells were pulsed with a p53 peptide corresponding to the product of a human lung carcinoma. Although transplanted spleen cells were pulsed with a high amount of peptide, *in vivo* generated p53 specific T cells also recognized and lysed cells with low expression of mutant p53 (0.18 ng/ml). These levels are comparable to levels of mutant p53 present in human tumors (0.1-70 ng/ml). Since mutations causing overexpression of p53 are present in a wide variety of cancers, a large group of patients would benefit from p53 directed immunotherapy.

One can consider mutant p53 sequences as target antigens for tumor specific CTLs. However, p53 mutations occur at different sites in the p53 molecule and it is necessary to identify the site of mutation in each patient before therapy. Furthermore, not all mutations are contained in MHC-binding CTL epitopes. If in contrast wild type p53 sequences are used, the entire sequence of the p53 protein is available for properly processed immunogenic T cell epitopes. We hypothesize that the altered expression of p53, seen in many cancers, might lead to modified processing and to the presentation of wild type derived peptides by MHC class I molecules. Indeed, recently wild type p53 peptide-specific CTLs were generated from human and murine responding lymphocytes and some of them recognized p53 overexpressing tumors *in vitro* (335-343). Vierboom et al. generated cytolytic T lymphocytes that recognized a murine wild type p53. Adoptive transfer of these CTLs into tumor bearing mice caused complete and permanent tumor eradication in the absence of any demonstrable damage to normal tissue. Wild type specific CTLs can apparently discriminate between p53 overexpressing tumor cells and normal tissue, indicating that widely expressed autologous molecules such as p53 can serve as a target for CTL-mediated immunotherapy of tumors.

Chapter I

Induction of cellular immunity by direct pDNA transfer of a mutated p53 allele into Balb/c mice

The principle of gene therapy is based on the introduction and expression of genes that compensate for the genetic defect that is responsible for the disease. A major intricacy for gene therapy of cancer is the problem to specifically introduce genes into neoplastic cells and by-pass normal cells. However, malign transformation is the result of a multiple genetic disorder and the introduction and expression of an individual gene as a therapeutic strategy is normally not sufficient. Therapy of cancer therefore requires the elimination of the neoplastic cells and not the compensation of a single genetic defect.

The mobilization of the immune system and the specific elimination of tumor cells are an approach being successfully used in the past. Experimental attempts are based on the insertion of the IL-2 or the IL-4 gene in tumor cells or surrounding mesynchymal cells and on the stimulation of a local immune response (344). Another immunological approach tested in mice is based on direct gene transfer of an allogeneic MHC-gene into tumor cells. A T cell dependent immune response could be induced due to still unknown tumor-associated antigens (345). Unfortunately these immunological principles are limited to cells of the primary tumor. We support the idea that new gene therapy protocols should embrace adjuvant treatment and the elimination of early metastatic cells (346). We want to develop gene therapy protocols that can be applied systemically. We describe different methods that sensibilize the immune system to tumor specific antigens and trigger an immune response that is able to eliminate tumor cells.

6.3 DNA-mediated immunization

In 1990, researchers from Vical (San Diego, CA) revolutionized the field of gene therapy with the startling report that injection of mice with purified plasmid DNA encoding a foreign protein resulted in abundant production of the protein (347-352). Within two years of discovery, several groups had demonstrated that injection with naked DNA vaccines led to the generation of long lived humoral and cellular immune responses against a range of antigens encoded by such plasmids, including proteins expressed by the influenza A virus, rabies virus, bovine herpes virus, hepatitis B and C, and HIV-1 (96, 102, 110-120). Viral infections primarily induce cytotoxic T cells because viral antigens mostly reside within the cell. It is now well established that these immune effector cells recognize antigenic peptides bound to MHC class I molecules on the surface of target cells (259-263). In parallel, tumor antigens resemble viral antigens, since they are synthesized within the cell and are uncommon for the cell type. The immune system is able to identify and to react to some of these tumor antigens, probably by the same immune effector mechanisms that are responsible for the elimination of virus infected cells. Indeed, different research groups successfully induced immune effector mechanisms that killed target cells "infected " by tumor antigens.

6.4 Analysis of pDNA transfer conditions

Different groups have shown that intradermal introduction and expression of DNA is possible and can induce effective cellular immune responses (354). We also could demonstrate that after pDNA injection the DNA encoded antigen was expressed at significant levels at the injection site. The transfer efficiency and the expression of the transgene strongly depended on the DNA buffer conditions. pDNA constructs mixed with PBS or complexed with a cationic liposomal transfection reagent proved to be most efficiently transferred. Cationic liposomes form complexes with the negatively charged DNA. This result in the presence of excess positive charge, which is required for an efficient interaction with the negatively charged cell membrane. We observed that the DNA : liposome ratio affected the gene expression in mice. 1 μg of pDNA mixed with 2 μl of the liposomal transfection reagent DOTAP showed best results. Additionally to the neutralization of the negatively charged DNA backbone, the protection of liposomal complexed DNA from protease cleavage in lysosomal compartments is of promising advantage. However, biolistic particle bombardment with gold particles coated with pDNA proved to be the most efficient gene transfer technology. Transfer of as little as 1 μg of pDNA resulted in expression levels ten times higher when compared to needle injection of 40 μg pDNA into the skin of mice. This method has been developed by Joel Hayes from Agracetus, who coated DNA onto thinly gold beads that were shot with a gene gun into the skin and introduced the DNA to epidermal cells.

Next we compared *in vivo* reporter gene expression upon needle injection using vector constructs with different strong viral promoter/enhancer elements. In general, reporter gene expression was highest at day two and three but strongly diminished within the first week. However, significant reporter gene expression was still detectable after two or three months providing a continuous antigen stimulus when used for immunization. Long term expression of the transgene has also been observed by various different groups (354-357). Our data corresponds to *in vitro* transfection assays performed by our group or the Tissue Engineering Center, Plastische Chirurgie, Universitätsklinik, Freiburg. However, we observed significant differences in reporter gene expression when it was controlled by different enhancer/promoter elements. Best results and highest expression was obtained when reporter gene constructs were expressed via the cytomegalovirus CMV promoter. Reporter gene expression was 10-15 times higher when compared to SV40 enhancer-promoter controlled constructs. We think that the induction of a protective immune response is favored by a persistent antigen stimulus and high expression of the transgene. For this reason cytomegalovirus CMV promoter controlled pDNA vaccines are most promising.

6.5 Localization of the transgene expression

For our *in vivo* experiments we planned to inject the pDNA vaccine into sites with high densities of antigen presenting cells. Of these, Langerhans cells as pivotal antigen presenting cells have been implicated in cell mediated immunity. Bergstresser et al. (358) determined surface densities of LCs at different rodent epidermal sites. High densities (900-1500 per mm^2) of LCs have been identified in the ears or food pads. We therefore think that the skin is an optimal target for pDNA application and for the induction of a cellular immune response.

Therefore we wanted to analyze β-galactosidase reporter gene transfer into the skin of mice. Upon intradermal injection we could demonstrate expression of the reporter gene in epidermal and dermal layers of the skin. Our results are in agreement with observations of Raz E.'s group, who showed that intradermal injection of pDNA leads to a prolonged expression of intracellular antigen by dermal keratinocytes, fibroblasts and cells with the morphology of Langerhans cells and macrophages (359). Dermal and epidermal layers are known to be a central region for immune responses. Dendritic cells, macrophages and Langerhans cells reside in these regions and serve as sentinels that encounter antigens following tissue damage or infection. After exposure to the antigen, inflammatory responses are induced, such as the production of cytokines and the activation of APCs. After antigen uptake and processing, APCs traffic to the lymphoid tissues were they localize to the T cell rich zones. Here, T cell priming proceeds upon delivery of at least two critical signals. The first signal confers antigen specificity through the engagement of the T cell's unique T cell receptor by peptide antigen presented on the major histocompatibility complex molecules of the APC. TCR signaling alone, however, is insufficient to prime naive T cells, a fact which is thought to prevent inappropriate activation of naive T cells that may encounter self-antigens on MHC molecules on normal tissue. The second non-antigen specific signal must be delivered to the T cell for full activation. The best-characterized source of a second signal is engagement of CD28 on T cells by its ligands CD80 (B7-1) or CD86 (B7-2) which are exclusively expressed on activated APCs.

We think that the migration of APCs to lymphoid organs is an important part necessary to induce an effective immune response. Trafficking of APCs to lymphoid tissue was demonstrated by injection of a luziferase reporter gene construct. After injection into the ears of Balb/c mice, we sectomized the animals and isolated the cervical lymph nodes. Cervical lymph nodes were analyzed for transgene expression by a standard luziferase assay. We could clearly demonstrate luziferase gene expression at day two post pDNA injection. We think that this might be due to the activation of APCs that upon antigen encounter migrate to neighboring lymphoid tissue to prime T and B lymphocytes. The timing is in accordance to the observation that the first wave of mitotic activity is normally seen in lymphoid tissue on day one or two after immunization. Another reason for reporter gene expression in neighboring lymph nodes might be due to the efflux of the injected solution. We observed that injected fluids disappeared within two hours after injection. Epithelia, such as the skin and the mucosa of the gastrointestinal and respiratory tracts have a lymphatic drainage. We think that pDNA molecules might be flushed from the injection site and transported to lymphoid tissue were they are sampled by lymph nodes after passage of the afferent lymphatics. Efflux of the injected fluids was demonstrated by injection of Isovit-300 and autoradiography. Independently on the mechanism how pDNA constructs are transported to the lymphoid tissue, the expression and processing of the transgene in lymph nodes meets one of the requirements for an effective activation of humoral and cellular immunity.

6.6 Protection from tumor growth by pDNA vaccination

It has been recognized for many years that tumors are often antigenic but not immunogenic (360, 361). Detailed analysis of tum$^-$ variants obtained by *in vitro* treatment with mutagens revealed that mice that had rejected tum$^-$ variants were significantly protected against a challenge with the original tumor (362, 363). This indicated that the latter was antigen positive. Since then it has been shown that many tumors express antigens which may be recognized by CTLs and thus display the necessary targets for potential immunotherapy.

In our model, pDNA immunization using a mammalian expression vector encoding a tumor antigen proved to be an efficient method to counteract tumor expansion *in vivo*. We chose the mutated p53 tumor suppressor antigen as a target for the induction of a protective immune response since it is frequently mutated and overexpressed in human cancers (6.2.1-3). Although most attention has been focused on the role of p53 in malignant transformation there is increasing interest in its ability to elicit cellular (364) and humoral immune reactions (365-367).

Different alleles of the mutated p53 tumor antigen were isolated from the murine fibrosarcoma cell line Meth A. These alleles have been reported to be able to induce cellular immunity by numerous groups (368-370). The Balb/c murine sarcoma Meth A is known for high p53 expression (371) and three missense point mutations in the p53 coding sequence (372). A nonamer peptide containing the codon 234 mutational product elicited peptide specific cytotoxic T cells and $CD4^+$ T helper cells. Immunization of mice with these peptides in adjuvant before tumor challenge inhibited Meth A tumor growth whereas immunization with peptides encoding the wild type sequence had no effect on tumor growth. Only the mutation in codon 234, resulting in a Met to Ile substitution, was found to create an antigenic peptide recognizable by CTLs, and only one of the peptides spanning this region (KYICNSSCM) generated $CD8^+$ T lymphocytes that lysed peptide pulsed cells in a K^d-restricted fashion. This peptide contained a tyrosine at position 2, a characteristic shared with other K^d restricted peptides (373). The corresponding wild type peptide (KYMCNSSCM) with the same motif did not elicit T cells. We think that the Met to Ile substitution in codon 234 (position 3 in the peptide) creates an aggretope rather than a new epitope, because isoleucine at position 3 in K^d-restricted monomers is a far more frequent residue then methionine (373), and X-ray analysis of HLA-B27 (374) and peptide motifs of mouse and human MHC molecules (375) suggest that amino acids at position 2, 3, 5, and carboxyl termini function as anchors or auxiliary anchors with their side chains in contact with pockets in the MHC molecule (Fig. 6.1).

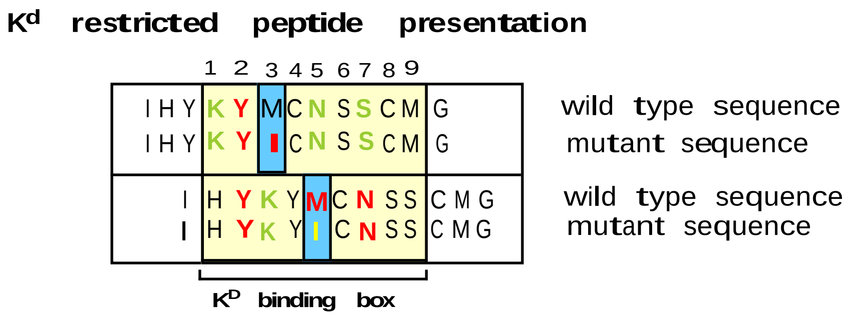

Fig.6.1 Anchor positions involved in K^d-restricted MHC binding. Peptides are bound as nonamers (yellow box). Tyrosine at position two is required for peptide MHC class I binding. Strongly binding (red) or weakly binding (green) amino acids within the pocket of the MHC molecule are illustrated by color. The wild type peptide and the mutant peptide, spanning the 234 mutation are described. The exchange of isoleucine for methionine at position 3 creates a new strong binding site for MHC class I molecules. However, exchange at position 5 results in loss of one strong binding site.

Noguchi et al. could even demonstrate the regression of established Meth A sarcoma transplants, when mice were vaccinated with the mutant peptide in combination of adjuvant and low doses of IL-12 (370). IL-12 is a strong inducer of IFN-γ (376) and facilitates the differentiation of T_H0 to T_H1 and T_H2 to T_H0 (377). However, the generation of synthetic patient-specific antitumoral peptides is expensive, time consuming and unrealistic on a large scale whereas antigen loading by gene transfer should be more efficient at CTL induction. pDNA vaccines are encoding the whole antigenic molecule that is processed to many different peptides, allowing determinant selection to occur. For clinical and veterinarian use, vaccines must be efficient across the diversity of many MHC haplotypes. The provision of whole protein rather than a limited number of peptides would therefore be advantageous. It is well established that tumors undergo antigenic variation and immunization with the full-length tumor antigen might be more effective instead of using only a single peptide antigen. pDNA vaccines also confer a long lasting antigen stimulus and we could demonstrate transgene expression for months. The use of peptides, on the other hand, is far more restrictive. They can activate just a single population of immune effector cells and are degraded much faster. For peptide pulsing, peptides have to incorporate specific amino acids with anchor residues that fit into the major grove of MHC molecules. In contrast to endogenously produced tumor antigens exogenous synthetic peptides should mainly target the MHC class II pathway: for example vaccination of patients with adenocarcinoma using mannosylated MUC-1 was efficient at stimulating antibody production and relatively inefficient at CTL induction (378).

6.6.1 Protection of immunized mice from solid tumor growth

For specific tumor cell lysis and immunization against an internal tumor antigen like mutant p53 we wanted to activate effector mechanisms that naturally recognize intracellularly altered cells. The induction of mutant p53 specific CTLs was performed by using mutant p53 encoding pDNA constructs. The pDNA was injected into epidermal layers of the ear or food pad of Balb/c mice, regions containing high surface densities of epidermal Langerhans cells (247), (6.5.1). After pDNA immunization mice were inoculated with tumor cells and tumor growth was analyzed. All non-treated control mice developed large tumors and died within 4 weeks. Mice injected with the pDNA vaccine, however, showed long termed resistance to Meth A tumor growth for at least 12 months. Time kinetics showed that protective immunity was induced most efficiently 2 months after onset of pDNA vaccination. pDNA immunization was carried out in 10 days intervals because *in vivo* luziferase reporter gene expression was significantly reduced after the first week (6.4). Immunization periods shorter then 60 days resulted in less efficient protection from Meth A tumor growth (> 20-30 days had no effect on tumor rejection). Injections of the pDNA vaccine did not cause any visible toxic effects and histologies from the injection site appeared normal. Neutralizing antibodies that bind to the plasmid molecules could not be detected.

pDNA vaccination was also performed by a gene gun. For biolistic particle bombardment pDNA was coated to gold particles and used for immunization. A protective immune response and Meth A tumor cell rejection was observed after 60 days and six immunizations. In general, immunization by injection of the pDNA vaccine as a liposome/pDNA mixture was more efficient than injection of pDNA solubilized in PBS. Biolistic gene transfer was least effective although reporter gene expression showed best results when using this method. Biolistic transfer is known to preferentially activate the Th2 subpopulation of T lymphocytes, whereas direct injection of naked DNA was connected to Th1 induction (379). The Th2 subset of lymphocytes is involved in the production of cytokines like IL4, IL5, IL6, and IL10 that promote B-cell activation and immunoglobulin class switching. However, type 1 like helper T cells support the development of cellular immune responses, including CTL and the IgG2a immunoglobulin subtype by production of cytokines like IL-2 and interferon-γ. Such phenomena might influence efficiency of tumor cell rejection, especially since it has been shown that the type of T cell help can have a profound effect on the outcome of a disease (380).

Next, we wanted to analyze the specificity of pDNA vaccination. We inoculated immunized, Meth A resistant mice with other syngeneic tumor cells. The growth of HC11-Q6 tumor cells was not influenced by pDNA-injection. The p53 gene in HC11-Q6 tumor cells is mutated at different regions and tumor cells harbor a phenotype with lower p53 expression levels. Therefore we think that the protection from tumor growth by the DNA-vaccine is most probably dependent on the presence of specific mutations within the p53 tumor antigen. Mice injected with pDNA encoding a bacterial β-galactosidase or a firefly luciferase gene were not protected against Meth A tumor cells. Again, this indicates that tumor-protecting properties are not due to the properties of the transfer plasmid-vehicle.

6.6.2 Established tumors cannot be reduced by pDNA vaccination

We also analyzed the therapeutic benefit of pDNA plasmids as an antitumor vaccine to reduce established tumors. Mice with transplanted tumors were treated with pDNA constructs but tumor growth was not influenced when compared to untreated control animals. Previous experiments showed that immune effector mechanisms were effectively activated 2 months after onset of pDNA-vaccination. However, tumors in unprotected mice already killed the animals within 1 month (immunization periods > 20-30 days had no effect on tumor rejection). Our experiments suggest that treatment of mice with established tumors did not allow the formation of an effective immune response in time and mice were killed by the tumor before the immune system could efficiently eliminate tumor cells or lead to tumor regression.
Another reason why pDNA vaccines were not efficient to reduce established tumors might be due to the fact that malignant cells often have immunosuppressive properties. It is now well established that solid tumors can secrete immunosuppressive factors that inhibit the activation of immune effector mechanisms. These factors might produce a microenvironment unfavorable for lymphocyte action and for other immune modulating cells. Indeed, North and Bursuker have distinguished two phases in the progressive growth of antigenic tumors (381). The initial phase from day one to seven was associated with the induction of $CD8^{+}$ T cells capable of transferring specific resistance to secondary donors. In the subsequent phase starting on day seven after tumor injection the protective T cell response disappeared and a $CD4^{+}$ cell population appeared that mediated specific suppression. In accordance with this observation we have found that intratumoral transfer of lymphocytes results in tumor regression but is loosing effectiveness depending on the size and age of the parental tumor (see 6.7.1).

6.6.3 Immunized mice are protected from pulmonary metastases

The treatment of disseminated metastases is still a most difficult task for the medical oncologist. The primary tumor can normally be excised by surgical means, but metastasis normally results in the death of the patient. Our next experiments examined the efficiency of pDNA vaccination to suppress metastases formation in a mouse model system. Meth A tumor cells form extensively pulmonary metastases upon intravenous transplantation into Balb/c mice. We analyzed survival of mice and the presence of disseminated tumor foci in different organs after pDNA vaccination and tumor cell inoculation. Histopathologic examination of sectioned tissue specimen demonstrated that pDNA-vaccinated mice did not display any tumors in the lung, spleen, kidney, and the liver. However, untreated mice showed disseminated tumor nodules that were infiltrating the pleura of the lung. Histopathologic analysis did not reveal metastasis formation in any other major organ of these mice. Additionally we evaluated the survival of mice post tumor cell inoculation. We observed that 100% of the non-treated animals died within 3 to 6 weeks because of lethal tumor growth, whereas all of the treated animals were distinguished by a disease free period for longer than 12 months. We conclude that pDNA vaccination efficiently protects mice from the development of pulmonary metastases. Resection of the primary tumor, followed by pDNA-vaccination against identified tumor antigens might prove as an effective means to protect the patient against a reoccurrence of the tumor or it might result in a prolonged disease free period of time after primary treatment.

6.7 The identification of effector mechanisms

Human tumors are poorly immunogenic although a large number of genetic alterations were found in advanced cancers and should give rise to peptide neoepitopes capable of being recognized by cytotoxic lymphocytes (33). Indeed, tumor-associated and tumor-specific antigens have been identified that could act as targets for immunological attack (34). It is therefore possible that the immune system fails to eliminate tumor cells not because neo-antigens are absent but rather because the response to these neo-antigens is inadequate (35). The induction of immune responses requires costimuli that may be cell surface molecules or specific cytokines. The B7 molecule, present on APCs is known to be a key costimulator for T cell activation (41-43). Experimentally, the presentation of MHC/peptide complexes to the TCR might lead to anergy in the absence of B7 costimulation. Therefore immunogenic tumors that do not express B7 can escape destruction by the immune response because tumor targeted T cells receive inadequate costimulation. Additionally tumors might lack or downregulate expression of MHC molecules or other molecules required for adhesion of lymphocytes such as LFA-1 and LFA-3 or ICAM-1 or they may express molecules like mucins that are anti-adhesive. They may also secrete immunosuppressive cytokines such as TGF-β. We analyzed the immunogenicity of Meth A tumor cells and determined cell surface expression of the costimulatory molecules B7-1 and B7-2 and the ability of tumor antigen presentation due to the presence of MHC class I and MHC class II molecules. FACS analysis clearly demonstrated MHC class I expression, but expression of MHC class II, B7-1, and B7-2 was missing. We think that the high tumorigenicity and low immunogenicity of Meth A tumor cells is based on loss of these molecules. In our model system tumor antigens can be presented via the MHC class I pathway but lack of expression of the other molecules results in the inability to induce a humoral or cellular immune response and therefore primary tumor cells in the non-vaccinated animal cannot be rejected.

We planned to induce protective antitumoral immunity in mice by the application of a pDNA based immunization that mimics, at least in part, the immune response induced by a viral infection. After incorporation of the vector constructs by professional APCs tumor antigen expression/processing is initiated and results in MHC class I presentation and the induction of cytotoxic T lymphocytes. In contrast to the naive T cell these cells are now independent of costimulatory signals and can perform active cytolytic activity upon MHC-antigen peptide encounter on the primary tumor cells. Principally, we expected cytolytic T cells to be the essential component of the antitumor effector mechanism in our model system, because recognition of Meth A tumor cells can only occur via the MHC class I pathway. An induction of T helper cells by the tumor seems unlikely since these cells need MHC class II presentation. However, the administration of pDNA has also proven to be an effective means of generating humoral immune responses specific for the transgene. Nevertheless, we do not expect B lymphocytes to play a major role in tumor rejection. First, the animals are immunized with a tumor antigen present within the tumor cell that cannot be detected or eliminated by secreted antibodies. In this case a humoral immune response seems to be inefficient. Second, tumor cells do not express MHC class II molecules and can therefore not induce T helper cells and the formation of a cytokine milieu that is essential for efficient B cell activation.

6.7.1 The cellular immune response

The first indication that cytolytic T lymphocytes might be responsible for Meth A tumor rejection was obtained by immunization studies using nude mice. These athymic mice lack the T lymphocyte dependent immune responses. In contrast to immunocompetent Balb/c mice these mice failed to develop protective immunity upon pDNA administration and tumor cell transplantation. We therefore think that the presence of T lymphocytes is a necessary requirement for tumor cell rejection. Direct evidence for the presence of mutant p53 specific cytolytic T lymphocytes in pDNA immunized Balb/c mice was obtained by a ^{51}Cr-release assay. We clearly demonstrated lytic potential of T lymphocytes using a dendritic cell line, transfected with mutant p53 constructs. On the other hand, lymphocytes isolated from untreated or tumor bearing mice did not show CTL activity. Our results clearly suggest that pDNA vaccination and expression of the transgene induces effectively CTLs that can eliminate mutant p53 expressing cells. Point mutations in the p53 tumor suppressor gene can create neo-antigenic determinants that serve as tumor antigens when processed and presented by class I MHC molecules (6.6). The specificity of the immune response was demonstrated by two experiments. First, non-transduced dendritic cells that did not express the mutant p53 genes were not lysed *in vitro* when incubated with lymphocytes isolated from Meth A resistant, immunized individuals. Second, Meth A resistant mice developed lethal HC11/Q6 solid tumors upon tumor cell transplantation. These tumor cells also express mutant p53 but contain different missense mutations.

The efficacy of our preactivated mutant p53 specific T cells was also tested in a T cell based cancer immunotherapy. It is firmly established in animal models that adoptively transferred specific T lymphocytes can eradicate otherwise lethal tumor loads (382-386). The success of this procedure is dependent on the number, the ability to home to target cells and the specificity of the transferred effector cells. We already could demonstrate that protection from Meth A tumor growth is mediated by cytolytic T lymphocytes in pDNA immunized mice. Further we wanted to investigate whether infusion of spleen derived lymphocytes from vaccinated animals into secondary tumor bearing recipients influenced progression of tumor growth. We could show that lymphocytes, sensitized by pDNA immunization, eradicated tumors or slowed down tumor growth when introduced intratumorally the mice. The adoptive transfer of these cells was capable of mediating complete regression of Meth A tumors within one week, observing first signs of tumor rejection two days post injection. However, these effects were not observed when transferred lymphocytes were derived from untreated or tumor bearing mice. Intratumoral or intravenous transfer of these lymphocytes mediated no delay in outgrowth of Meth A tumors. Macrophages also did not seem to be involved in the process of tumor rejection. When isolated from the peritoneum of pDNA-immunized mice and injected into tumor bearing recipients, they had no effect on tumor growth.

In general, the effect of tumor regression was dependent on the size of the tumor and the timepoint of lymphocyte transfer post tumor cell inoculation. Growth of large tumors was not influenced by T lymphocyte transfer. We think that the amount of injected Meth A reactive lymphocytes and the number of transfer infusions might be crucial for successful tumor eradication. The implication of a well-balanced ratio of CTLs and tumor mass is reflected by the observation that lymphocyte transfer can induce temporary growth inhibition for about 10 days. Successive CTL-immunizations might revert tumor growth and lead to the eradication of malign tumor cells. However, the treatment of large tumors might also be complicated by secretion of immunosuppressive factors and inhibition of immune effector mechanisms. Similarly, North and Bursuker have distinguished two phases in the progressive growth of antigenic tumors (381). The initial phase is associated with the induction of $CD8^+$ T cells capable of transferring specific resistance to secondary donors. In the subsequent phase starting seven days post tumor injection the protective T cell response disappears and a $CD4^+$ cell population appears that mediates specific suppression. Indeed we speculate that immunosuppressive events might be involved when tumors are not rejected following lymphocyte transfer. Our hypothesis is founded on the observation that pDNA vaccination is also inefficient in mice with established tumors (see 6.6.2). In accordance with this view intratumoral transfer of lymphocytes isolated from immunized mice might very well loose effectiveness depending on the size and age of the tumor.

Our results clearly demonstrate that the adoptive transfer of spleen-derived lymphocytes from pDNA-vaccinated mice transfers antitumor immunity to non-vaccinated recipients. This indicates that CTLs are the predominant effector cells.

Cured mice stayed tumor free for a follow up period of 12 months. However, intravenous administration of T lymphocytes showed no antitumoral effect, maybe due to an inappropriate amount of transferred CTLs or an inappropriate homing of *in vitro* cultured lymphocytes. We think that our results might be improved by increasing the number of transferred lymphocytes or by repeated adoptive transfer of CTL effector cells, especially in chases where one single lymphocyte injection resulted in temporary tumor growth inhibition.

6.7.2 The humoral immune response

Administration of pDNA has proven to be an effective means of generating humoral immune responses specific for the transgene (387, 388). The activation of B lymphocytes can be demonstrated by the presence of specific serum antibodies following pDNA vaccination. The antibody isotypes induced by pDNA immunization are generally IgG, but serum IgM and IgA also have been detected by different groups (388, 389). In mice the subclass of serum antibodies induced by DNA-vaccination is predominantly IgG2a suggesting that the generation of Th1-like T cell help may be a general property of DNA-vaccines. However, vaccination using a gene gun appears to shift immune responses toward Th2-like responses that is typified by a predominance of the IgG1 immunoglobulin isotype (390). The identification of the antibody isotypes therefore might give a clue which T helper subtypes are predominantly induced by our pDNA vaccination technique. This is particularly important because dependent on the type of T helper cell activation different functional subsets of immune reactions can be distinguished. For mice, a predominance of the IgG2a isotype indicates activation of type 1-like helper T cells that produce cytokines such as IL-2 and IFN-γ, and support the development of cellular immune responses, including CTLs. On the other hand cytokines like IL-4, IL-5, IL-6, and IL-10, are produced by type 2-like T helper cells, that promote B cell activation and immunoglobulin isotype switching. Activity of Th2 cells is indicated by predominance of the IgG1 immunoglobulin isotype.

We wanted to identify serum antibodies recognizing mutant p53 using a Western blot procedure and examined binding to recombinant mutant p53. Different groups of mice were analyzed: pDNA vaccinated tumor resistant mice that were immunized by injection or particle bombardment; untreated control mice; tumor-bearing untreated control mice; tumor-bearing pDNA immunized mice. Serum samples were taken from the lateral tail vein pre-study, post immunization, and after tumor cell inoculation. None of these mice displayed antibodies binding to recombinant mutant p53. The absence of a humoral immune response is not surprising. We did not expect the humoral immune response to be efficient for tumor cell rejection because of three reasons. First, immunization of nude mice with our pDNA constructs proved to be inefficient and mice succumb to lethal tumor growth. Athymic nude mice are characterized by a phenotype negative for T lymphocytes although B cell populations are unaffected in these animals. Second, p53 is an intracellular protein that under normal conditions is not accessible for circulating antibodies. Serum antibodies only recognize peripheral antigenic structures. Third, second messenger activation of B lymphocytes is often provided by contact with helper T lymphocytes and by cytokines produced by these cells. It is now well established that the pDNA is taken up by various cell types of the skin which then produce the gene product (391). Therefore mutant p53 becomes an intracellular antigen that can be processed and presented via the MHC class I pathway like other intracellular antigens (392). This pathway is known primarily to induce cytolytic T lymphocytes that can eliminate infected cells. Additionally tumor cells do not express MHC class II molecules and are not able to support T helper cell activation. Thus, our results clearly suggest that a humoral immune response does not contribute to tumor rejection.

6.8 Optimization of an antitumor immune response

Immune responses generated by pDNA vaccination can be optimized by the codelivery of costimulatory molecules like CD80 or CD86 (393). The CD80 (B7-1) and CD86 (B7-2) molecules interact with the CD28/CTLA4 molecules on T cells and provide an important second signal in addition to ligating the T cell receptor to the MHC peptide complex (394, 395). These two signals then facilitate expression of IL-2 receptor and progression of the T cell through the cycle of immune activation. CD80 and CD86 are upregulated during the antigen presentation, most likely following CD40/CD40-ligand interactions between T cells and APCs (396). Indeed, it could be shown that the coadministration of the CD86 gene resulted in a dramatic increase in cytotoxic T lymphocyte induction as well as T helper cell proliferation. Other reports describe that coinjection with plasmids encoding cytokines, such as granulocyte-macrophage colony-stimulating factor, enhances antibody production and T helper responses to the targeted antigen. In contrast, coinjection with plasmids encoding IL-12 blunts the antibody response but enhances T helper and CTL responses (397, 398). Based on this results we support the idea that the delivery of genes encoding immunomodulatory molecules together with those that encode the antigen permits to boost the immune response and allows the tailoring of the immune response toward effector mechanisms that are best suited for an antitumor immune response.

6.9 Evaluation and future aspects of pDNA vaccines in antitumor immunity

Part of the appeal of DNA vaccines is that in contrast to the more traditional methods vector constructs offer a number of attractive attributes: their simplicity, the apparent robustness of the technology, their efficacy and applicability to various pathogens and diseases and their ability to induce vigorous cellular immune responses. They have also been shown to break haplotype restriction and individuals nonresponsive to the recombinant protein-vaccine suddenly generated H-2K^b and H-2D^b-restricted CTL responses when immunized by the pDNA vaccine (399). The efficacy of DNA vaccines can be enhanced or modulated through the use of formulations that increase DNA stability, the coexpression of immune molecules that affect the processing of antigens, or through the use of adjuvants that affect the immune responses that are mounted against the expressed antigen. Another potentially effective and simple way of enhancing immune responses to pDNA vaccines is via an adjuvant effect of the DNA itself. Work over the past five years has shown that certain sequences can induce cytokine secretion and lymphocyte activation (400, 401). It has been shown that certain CpG motifs in bacterial DNA are particularly stimulatory, probably due to the methylation state of the DNA (402).

One requirement for successful pDNA vaccination is the presence of an intact immune system. From a clinical point of view, the treatment of human cancer is only possible if it is assumed that human tumor cells can be specifically recognized by immune effector mechanisms. For specific antitumor immunity tumors must express tumor antigens and accessory molecules that can be recognized by cells of the immune system. Point mutated oncogenes are not likely to be presented since the estimated chance for successful association of such a peptide given a certain set of MHC haplotypes is low (403). The deletion of tumor suppressor genes is not at all expected to betray itself in this way. In addition, there is evidence to suggest downregulated MHC class I expression as a fairly common mechanism to escape from immunosurveillance (404, 405). Thus we must face the possibility that many tumors do not express cell surface structures that can trigger an antitumor immune responses. In our case the analysis of Meth A tumor cells showed no expression of the costimulatory molecules B7-1 and B7-2 and no expression of MHC class II molecules. On the other hand these tumor cell express MHC class I molecules that are necessary for the induction of cytolytic T cells. We strongly suspect that Meth A tumor cells are immunogenic, but are not rejected by the host animal because in contrast to preactivated T cells naive T lymphocytes miss the contact with costimulatory ligands beside encounter of an antigenic MHC-peptide complex (6.7). By pDNA delivery encoding defined immunogenic tumor antigens, professional antigen presenting cells can take up the pDNA vaccine and present immunogenic peptides in combination with the required costimulatory molecules to the immune effector cells of the host. Indeed we could only identify MHC class I associated effector mechanisms in immunized individuals (6.7.1).

Unfortunately our mouse model system is unsuitable to examine pDNA vaccines as a means of tumor therapy. We observed that Meth A tumors are extremely lethal and kill mice within one month when applied subcutaneously or even after two to three weeks when metastasis formation is induced. On the other hand protective immunity needs two months to develop effectively, a common observation in pDNA vaccination (406). We observed that a subsequent administration of the pDNA vaccine in three day intervals post tumor cell inoculation did not influence the development of the tumor and corroborate the importance of time the individual needs to set up an effective immune response. However, our model was extremely effective to demonstrate the induction of a protective antitumor immune response *in vivo*. The data obtained in our mouse model system is of direct relevance to the treatment of human tumors. We recommend the use of pDNA as an antitumor vaccine to immunize individuals with inherited disorders that predispose to cancer. The observation of the familiar environment might give a clue for individuals that run a higher risk to be affected by this disease. Familiar disposition to cancer is often associated with acquired genetic disorders. Another application of our vaccine might result in the systemic treatment of patients post operation and chemotherapy. After surgical resection of the primary tumor and chemotherapy, patients often succumb to a disease free period for some years before reoccurrence of the tumor. Reoccurrence of the tumor is often associated with disseminated tumor nodules, higher aggressiveness of tumor cells and resistance to chemotherapy and often results in the death of the patient. We hope that a pDNA immunization during this period can induce protective immunity targeted for cells that show the typical genetic disorders and might result in a prolonged disease free period or even in the cure of the patient. Our pDNA vector constructs encoding mutant p53 proved to be specific for typical genetic disorders and did not act on tumor cells expressing different mutations. Our experiments also clearly demonstrated no relapse of Meth A resistant mice, even after repeated tumor cell inoculation. The formation of metastases was totally suppressed in these mice.

However, one should not forget the risks involved in the application of pDNA vaccines. Beside the concerns of inducing autoimmune diseases when using pDNA vector constructs encoding neoantigens, the introduction of tumor antigens into normal cells might result in the deregulation of normal cell function. The p53 tumor suppressor is known to be a central player to prevent tumorigenesis, active as a tetramer. It is now well established that mutant p53 can act in a dominant negative manner by oligomerization with wild type p53. The introduction of a mutant allele can therefore eliminate the tumor suppressive properties of wild type p53 and might result in tumorigenesis. This can be prevented by using pDNA constructs encoding mutant p53 fragments that have lost protein oligomerization function and DNA binding properties by deletion or specific point mutation. However, the main source of unease among scientists and clinicians is the possibility of random integration of plasmid DNA into the host genome with an attendant risk of mutagenesis. If such insertional mutagenesis takes place this could result in the activation of cellular proto-oncogenes or the inactivation of some tumor suppressor genes. Alternatively, insertion into germ cell DNA could cause genetic disease in a future generation. These concerns are particularly justified if one is eventually to consider vaccinating large populations of otherwise healthy individuals, especially infants or children. If there is a real risk of insertional mutagenesis with pDNA, then the potential benefits of prophylactic immunization obviously cannot justify a perceived increased risk of mutational events. This fear of the use of pDNA arises particularly from the well-known ability of certain viruses to insert their nucleic acid into the genome of the infected cell. However, the vectors used for immunization do not need to contain any sequence capable of promoting integration. The high level of integration of viral genomes is, after all, an active process requiring accessory cellular enzymes, such as helicase, topoisomerase or integrase. Nonetheless, low levels of integration of pDNA can be obtained in cell culture under optimized conditions. However, *in vivo* studies showed that injected pDNA constructs remained extrachromosomal although they remained in the tissue for weeks or even months and could be expressed for long periods of time (407). In addition, terminally differentiated cells are normally in a postmitotic state, and the absence of cellular DNA replication in the cells that have taken up the DNA will guard against integration that is thought to normally require a round of DNA synthesis.

Chapter II

Transduction of dendritic cells with mutant p53 alleles creates an efficient antitumor vaccine

The description of skin dendritic cells by Langerhans in 1968 was followed by prolonged speculation as to their function. Steinmann and Cohn identified mouse spleen DCs in 1973 (408) and initiated a series of experiments that established lymphoid tissue derived DCs as potent stimulators of primary immune responses (409-411). However, studies of DCs have been greatly hampered by their low frequency in blood and tissue and by lack of specific DC markers. Nonetheless, several laboratories persisted with their investigation leading to the current acceptance that DCs represent discrete leukocyte populations that arise from $CD34^+$ progenitors in the bone marrow and exhibit typical functions of professional antigen presenting cells (412, 413). Along with B cells and mononuclear phagocytes these cells are capable of highly efficiently present antigens to the immune system in the context of both major histocompatibility complex class I and class II molecules. What makes DCs stand out from other professional APCs, however, is their seemingly unique ability to present antigen to T lymphocytes which have had no previous contact with antigen and induce primary immune responses, i.e., activate immunologically naive T cells (414). This gives DCs a central role in the initiation of immune responses and creates possibilities for their use in the development of therapeutic strategies against tumors and other diseases.

However, a paucity of markers for DCs, the difficulty distinguishing DCs from monocytes/macrophages, and the problems involved in purifying DCs slowed down their use as anticancer agents. Current approaches for the enrichment of mouse DCs which are present at very low frequencies in lymphoid and non-lymphoid tissue as well as in the circulation (415-419), are usually difficult and time consuming multistep procedures. Generally, knowledge about DCs has been obtained mainly from studies of DCs enriched by density centrifugation, followed by over night culture and negative selection (420-424). We used a faster and more convenient way for the enrichment of primary DCs from mouse spleen or bone marrow. CD11c, MHC class II positive DCs can be obtained using positive selection columns. The method is based on the positive selection of mouse DCs with MACS CD11c MicroBeads. The CD11c antibody clone N418 is specific for the α^x subunit of the leukocyte integrin $\alpha^x\beta^2$ (425). CD11c is strongly expressed on mature mouse DCs from lymphoid tissues (426) and on $CD4^-/CD8^+$ intraepithelial lymphocytes (427). The CD11c antibody is labeled with magnetic beads and is retained in a magnetic field separator. For isolation of primary DCs, cell suspensions were incubated with MACS CD11c MicroBeads and passed over a magnetic field seperator. Isolated cell fractions were subjected to FACS analysis, and DCs were identified by anti-CD11c-PE and anti-MHC class II-FITC staining. Before seperation, bone marrow derived cells were cultured in the presence of IL-4 and GM-CSF for 7 days to differentiate $CD34^+$ progenitors into fully developed DCs. MACS separation enriched $CD11c^+$/MHC class II^+ cells from 15% to 53%, with a total yield of 1 x 10^6 DCs/mouse. On the other hand, spleen cell suspensions contained 47% $CD11c^+$/MHC class II^+ cells before separation. After MACS, spleen derived DCs were of higher purity (86%) and higher yield (2-4 x 10^6 DCs/spleen) when compared to bone marrow derived DCs. Bone marrow derived DCs and spleenic DCs did not only vary in purity of cell fraction and number of positive cells but we also think that they might be characterized by differences in antigen uptake and ability to stimulate immune responses. It has been shown that bone marrow isolated from mice does not have constitutive allostimulatory activity (428). However, mouse bone marrow cultured in GM-CSF and IL-4 enables a DC-like population to emerge (429-432). These generated mouse DCs are alloresponsive but lack significant expression of mouse DC markers like N418 (430). This fact explains the low amount of isolated bone marrow derived DCs. On the other hand, spleenic dendritic cells express CD11c, high-density MHC products, low level CD4, and certain CD14 epitopes detected by some monoclonal antibodies (433, 434). It is now well established that spleenic DCs are potent APCs in allo-MLRs (435-437) and oxidative mitogenesis assays (438). In our next experiments we tested primary DCs isolated from Balb/c mice in an alloresponse using purified T lymphocyte populations from C57/BL6 mice. Results were compared to the induction of an alloresponse by the dendritic cell line D2SC-1. This cell line has already been described as very efficient stimulators of naive or pre-sensitized T cells (278). Indeed, ^{3}H-incorporation into T cells was comparable when using enriched bone marrow derived DCs, spleenic DCs or the dendritic cell line D2SC-1 as target cells. Bone marrow has been described as week stimulator in an allo-MLR by different groups (439, 440). However, we could show that cytokine activated dendritic cells, differentiated from bone marrow, gained significant allo-MLR activity when treated with GM-CSF and IL-4.

6.10 Transduction of dendritic cells with tumor antigens

We wanted to transduce DCs with tumor antigen and use them as cellular vaccine to induce antitumoral immune responses. However, transfection of DCs with tumor antigens and their ability to induce an antitumoral immune response *in vivo* might depend an the maturation stage (446). During development of a dendritic cell the cellular machinery is switched from antigen processing and presenting to T cell costimulation and activation (441-445). The maturation stage might therefore influence transfection efficiencies or antigen uptake, processing and presentation of the antigen, clustering and activation of T and B lymphocytes and costimulatory properties. We were able to isolate bone marrow derived primary DCs and spleenic mature DCs. Additionally we used a DC derived immortalized cell line D2SC-1 that was already described to be a efficient for T cell activation, as shown in an alloresponse.

Different methods were used to transfect DCs, such as calcium phosphate precipitation, lipofection, electroporation and retroviral transduction. Transfection efficiencies were compared by GFP- or β-Gal reporter gene expression. Renca cells that were used as a positive control for successful transfection showed nice expression of the reporter genes. On the other hand we were unable to visible transfect primary dendritic cells. Retroviral transduction, calcium phosphate precipitation or lipofection of primary dendritic cells was totally inefficient,

whereas electroporation resulted in just a few positive cells. These results match with published data describing gene transfer to primary dendritic cells only in combination with the most sensitive bioactivity assays (447, 448). So far we could not find any publication, demonstrating direct efficient gene transfer into these cells. We think that one obvious reason for the low transfection efficiency is due to the fact, that terminally differentiated, non-deviding cells as primary dendritic cells are generally difficult to transfect. However, recently new transfection protocols using adenoviral gene transfer have been successfully established (449-454). Adenoviral infection and gene transfer is independent of mitosis in contrast to retroviral gene transfer that needs cell division and nuclear breakdown for the integration of the viral DNA into the genome. Therefore this new method might be useful to transfect primary dendritic cells in the near future. Another possibility for efficient gene transfer might be the transfection of $CD35^+$ positive progenitor cells in the bone marrow before they differentiate to functional dendritic cells. Successful transfection still has to be demonstrated.
The use of the dendritic cell line D2SC-1 for gene transfer proved to be more successful. Retroviral transduction of the dendritic cell line D2SC-1 was very efficient in our hands. By repeated infection with β-galactosidase gene containing retroviruses we obtained 70-100% transduced DCs. In the following experiments this method was used to transfer the mutant p53 tumor antigen into the antigen presenting dendritic cell line D2SC-1. This cell line was then used as targets for *in vitro* ^{51}Cr release assays or for induction of *in vivo* antitumor immune responses.

6.10.1 Use of transduced DCs as targets cells for cytolytic T lymphocytes

We wanted to use the antigen presenting properties of transduced dendritic cells to screen spleenic lymphocytes of immunized mice for cytolytic activity in a ^{51}Cr release assay. Transduction of the dendritic cells with the p53 tumor antigen was performed by a stably transfected retroviral packaging cell line TeFly AF13 (234). Stable clones budded mutant p53 positive virus transfer vesicles into the supernatant. The retrovirus containing supernatant was used for infection of DC target cells. The virus titer was determined to be 2.4×10^5 CFU/ml. Multiple infections enlarged the proportion of transduced target cells. DCs, transduced by the tumor antigen were assayed by FACS analysis and analyzed for p53 expression. Again, 70-100% of dendritic cells were positive and showed enlarged expression of p53. Subsequently these cells were labeled by incorporation of 51-sodium chromate and used as target cells for a chrome-release assay. In parallel, effector cells were isolated from mutant p53 pDNA immunized mice or untreated mice. As specified by release of sodium chromate into the supernatant, cytolytic T lymphocytes, isolated from pDNA immunized mice clearly recognized transduced DCs. On the other hand, T cells from untreated mice did not recognize the p53 tumor antigen and did not show cytolytic activity within four to six hours.

6.10.2 Use of transduced dendritic cells as a cellular vaccine

Protocols for clinical immunotherapy programs, targeted on malignant cell antigens or infectious agents were designed to exploit DCs as nature adjuvants for an optimal therapeutic vaccination. Because DCs can prime animals in the absence of any other adjuvant they have been termed nature's adjuvant. DCs express high levels of antigen presenting major histocompatibility complex products (HLA-DP, DQ, DR; HLA-A, B, C) as well as several accessory molecules (B7-1, B7-2, LFA-3, ICAM-1, ICAM-3, CD40) that mediate T cell binding and costimulation. These properties combined with the presentation of an immunogenic tumor antigen might present a potent cellular vaccine to protect the vaccinated individual from tumor growth. In mice, protective immune responses against tumors can be induced after immunization of the animal with DCs pulsed with defined synthetic tumor peptides (455), acid-eluted peptides from tumor derived cell lines (456), intact soluble tumor proteins (457), or with DCs transfected with tumor derived genes (458, 459). One advantage of using pDNA transduced DCs instead of peptide pulse DCs is that the pDNA encodes multiple epitopes for many MHC class I alleles. Hence pDNA transfected DCs can be used to stimulate CTL responses in many different individuals without prior knowledge of the haplotype of the individual. By using the whole gene we also avoided the need to know which specific epitopes of the tumor antigens can induce cellular immune responses, a fact necessary to know for synthetic peptide design prior DC pulsing.

We tested a new strategy for cancer immunotherapy by using mutant p53 transduced dendritic cells as a cellular vaccine. Their potential to induce protective immunity in mice when injected into the animal was analyzed. Upon intravenous or subcutaneous injection we demonstrated that DCs transduced with RNA encoding the mutant p53 tumor antigen were potent immune stimulators that can induce protective immunity in mice. After tumor cell inoculation, 50% of treated animals were resistant to Meth A tumor cell growth and the formation of metastases. Protection from tumor development was independent from subcutaneous or intravenous application of the cellular vaccine. Interestingly, both mutant p53 alleles developed a protective immune response in the vaccinated animal.

Our transduced dendritic cells were clearly able to induce cytolytic activity of T lymphocytes when isolated from pDNA vaccinated animals (6.7.1), a necessary requirement for the induction of an effective antitumor immune response. However, the presence of mutant p53 specific T cells in DC vaccinated animals still has to be established. Generally, the induction of a cellular immune response can be analyzed using standard proliferation assays, the specific cytolytic T cell activity can be demonstrated by a ^{51}Cr release assay. Alternatively *in vivo* depletion studies using anti-CD4 or anti-CD8 antibodies can be invented to eliminate single effector populations by specific complement activated lysis. Corresponding experiments are ongoing.

6.11 Tumor cells can escape immunotherapy

Tumor escape mechanisms are a common phenomenon in cancer. Mice that developed normal Meth A tumors were killed within one month and explanted tumor cells showed a Meth A p53 specific phenotype. However, we observed that in some tumor resistant mice tumors developed spontaneously after about three months. Those tumors were excised and analyzed for p53 expression. Interestingly, these tumors had downregulated their p53 levels as shown by FACS analysis. We think that tumor cells might have escaped detection from the immune system by clonal expansion of cells with low or undetectable levels of mutant p53. We could also show that the immune response, induced in the vaccinated animal was based on specific mutations within the p53 tumor suppressor gene. Immunized, tumor resistant mice injected with the ras-transformed mammary epithelial tumor cell line HC11-Q6 developed tumors and eventually died. The same phenomenon was also observed in 6.6.1.
We conclude that the treatment of a large variety of tumors depends on the isolation of mutated genes and the identification of specific immunogenic mutations encoded by these tumor antigens. We also conclude that upon specific immunization of individuals with tumor antigens a downregulation of these genes as apart of a tumor escape mechanism has to be expected. However, in our case downregulation of p53 might make tumor cells more susceptible to chemotherapeutic treatment, were resistance has been observed in combination of p53 mutation and overexpression (6.2.2).

6.12 Future aspects

The dendritic cell network is a specialized system for presenting antigen to naive or quiescent $CD4^+$ or $CD8^+$ T cells (462). Numerous studies have documented the exceptional ability of DCs to stimulate T cell responses *in vitro* and *in vivo* (463-469). We demonstrated that dendritic cells transduced with mutant p53 are potent inducers of a protective antitumor immune response in vaccinated mice. Tumor rejection was specific for Meth A tumor cells since the ras-transformed mammary epithelial tumor cell line HC11-Q6 formed lethal tumors on the vaccinated animal. Interestingly, *in vitro* p53 transduced DCs only induced preactivated T lymphocytes isolated from the pDNA immunized animal, whereas naive T cells could not lyse the mutant p53 expressing target cells (6.7.1). This might be due to the fact that a naive T cell needs a predisposition phase for activation and cannot directly lyse its target cells. In our ^{51}Cr-release assay the naive T cells were exposed to the tumor antigen for about six hours, obviously not long enough to get them activated and to perform cytolytic activity. Standard *in vitro* activation of naive T cells normally takes some days in cell culture.
In our next experiments we want to identify the effector mechanisms in the immunized animal that are responsible for tumor cell rejection. Generally, the induction of T lymphocytes can be analyzed using standard proliferation assays and the specific activation of cytolytic T cells can be measured by a ^{51}Cr release assay. Alternatively, *in vivo* depletion studies with anti-CD4 or anti-CD8 antibodies can be used to eliminate single effector populations by specific complement activated lysis. The presence of mutant p53 specific antibodies will be analyzed by a Western blot experiment. Corresponding experiments are ongoing.

Our experiments point out that the use of transduced, tumor antigen expressing DCs can successfully induce protective antitumor immunity. However, we did not test efficacy of the cellular vaccine to cure individuals with established tumors. Previous experiments showed that Meth A tumors are fast growing and lethal within one month. Therefore we speculate that according to experiments performed by pDNA vaccination the tumor-bearing host is unable to develop protective immunity in time. According to the conclusions described in 6.6.2, we speculate that the use of immunotherapy post surgical resection of the primary tumor might be advantageous. Normally patients underlie a disease free period after removal of the primary tumor before reoccurrence of the malignancy. This period might be used to develop an efficient antitumor immune response that can eliminate remaining malignant cells and avoid or suppress the formation of disseminated metastases, the main reason for death of cancer patients.

Nevertheless, the use of transduced dendritic cells as a cellular antitumor vaccine is problematic. The presentation of self-proteins representing tumor antigens can induce autoimmune responses with pathological consequences or induce tolerance. We did not observe signs of autoimmunity in any treated animals. This corresponds to the observation of other groups. No evidence of autoimmunity was seen in animals using non-fractioned tumor material as a source of tumor antigens (460, 461). However, the development of increasingly potent vaccines may very well lead to some autoimmune manifestations.
Most studies focus on the power of DCs to activate T cells but before T cells encounter foreign antigens the T cell repertoire is tolerized to self-antigens. This occurs in the thymus by deletion of developing lymphocytes and in lymphoid organs probably by the induction of anergy or deletion of mature T cells. In both cases the DC system is involved in tolerizing T cells to self-antigens. In the thymic medulla DCs present self-antigens via MHC molecules and thymocytes that have a too high affinity for self-antigens are deleted. If antigen bearing DCs are directly injected into the developing thymus reactive lymphocytes are deleted. On the other hand, if MHC class II molecules are only expressed by cortical epithelium and not by DCs in the medulla,

the possibility to develop autoimmunity increases, indicating that DCs in the medulla are responsible for the deletion of autoreactive cells (470).
Recent studies point to an important role for DCs in the induction of peripheral tolerance as well. DCs can capture and present self-antigens that are exclusive to specialized tissue. For example, bone marrow derived APCs present peptides that are derived from insulin producing β-cells of the pancreas to T cells in the draining lymph node. Tolerance ensues, probably as a result of T cell anergy or deletion (471, 472). DCs are also able to present many self-antigens due to normal turn over of somatic cells and thus induce tolerance to self-proteins that have no access to the thymus (473). It is yet not known what determines if DCs are turning on or off the immune system. DCs are long lived and express high levels of MHC-antigen complexes (474) and maybe T cells become anergic or die in response to abundant and persistent antigens. Maybe distinct DCs are responsible for the distinct task and tolerance inducing DCs are qualitatively different, perhaps expressing death molecules like the fas ligand (475).

Chapter III

Generation of an effective cellular antitumor vaccine by fusion of professional antigen presenting cells with fibrosarcoma cells

The idea of immunotherapy as a new strategy for the treatment of cancer is characterized by renewed optimism, largely based on a better understanding of how cytolytic T lymphocytes respond to tumor cells (476-478). Many tumor components do not elicit an antigen specific T cell response in patients which may be due to the absence of functional antigen presentation and secretion of factors such as IL-10, TGF-β, and VEGF that reduce DC development and function. It is well established that the immune repertoire carries tumor reactive lymphocytes, especially CTLs, but there is little evidence that these cells are being activated *in vivo*. However, when tumor antigens are applied to DCs *ex vivo* and these DCs are then reinfused, specific immunity ensues. In animals this strategy can lead to protection against tumors and even to a reduction in the size of established tumors (479-481). DCs can readily elicit helper and killer T cells, antibodies and IL-12. In contrast, many existing vaccines and adjuvants are week stimulators of $CD8^+$ T cells and Th-1 type T cells. The potency of the immune response that is elicited by dendritic cells is also impressively illustrated by their ability to break neonatal tolerance (482). In a host of experiments over the past years, dendritic cells have been used to process and present tumor antigens. Tumor antigen presentation has generally been achieved by pulsing cultured cells with peptides eluted from class I MHC molecules (483), tumor cell membranes (483) or RNA derived from the neoplastic cell (484). We have pursued an alternative strategy of introducing tumor antigens into dendritic cells by fusing entire tumor cells with cultured dendritic cells.
When cells from a murine fibrosarcoma cell line were fused with the dendritic cell line D2SC-1 we obtained fusion hybrid clones. The use of dendritic cells as a fusion partner offers three advantages. First, tumor antigens are combined to antigen presenting properties of DCs. Second, dendritic cells are known to prime naive CTLs and third, DCs are easily accessible from peripheral blood or bone marrow. For selection of fusion cell clones tumor cells and DCs were stably transfected with antibiotic resistance markers. After addition of selective medium only the cell fusion clones survived. As a safety precaution, we suppose to cotransfect tumor cells with inducible suicide gene encoding constructs (e.g. herpes simplex virus-thymidin kinase, cytosine deaminase, deoxycytidine kinase (485)) and tumors and tumor fusions can be eliminated when undesirable anti-self responses emerge in the immunized individual.

After cell fusion, surviving cell clones were analyzed for the expression of MHC class I, MHC class II, B7-1, and B7-2 molecules. Meth A tumor cells expressed only MHC class I molecules, whereas the dendritic cell line D2SC-1 additionally was positive for MHC class II, B7-1 and B7-2. We think that the combination of various tumor antigens with the antigen presenting properties of DC function should generate a powerful cellular vaccine. However, FACS analysis showed that the single fusion clones showed different levels of expression of these molecules (see appendix). In the following experiments we tried to analyze the efficacy of individual clones to induce protective immunity in the vaccinated animal and connect immunity to the expression of MHC- and costimulatory molecules. The fusion cell hybrids were used to immunize syngeneic mice and generated a strong antitumor immune response. This antitumor activity both actively seemed to involve humoral and cellular immune mechanisms. However, we were not able to connect cell surface expression of MHC class I, MHC class II, B7-1 or B7-2 to antitumor immunity. The vaccinated animals were protected from primary tumor growth independently on cell surface expression of these molecules. More excitingly and potentially of greater clinical significance, this antitumor activity occurred not only against the primary tumor, but also against metastasis formation. When injected separately into the syngeneic animal, Meth A tumor cells or the dendritic cell line D2SC-1 formed lethal tumors. However, in vaccinated Balb/c mice, fusion clones formed palpable tumor nodules that were eliminated within seven to ten days. We suspect that cell fusion clones loose tumorigenicity and gain immunogenicity due to the fact that tumor antigens are combined with antigen presenting properties. More importantly, the immune response is obviously induced in a very short period of time. We therefore support the idea to use this vaccination technique to treat established tumors or metastases in our next set of experiments.

6.13 Generation of a humoral immune response

Dendritic cells have a major impact on the regulation of immune responses. They are known to influence B cell growth and immunoglobulin secretion. DCs activate and expand Th cells, which in turn induce B cell growth and antibody production. By secretion of soluble factors including IL-6, IL-12, and TNFα DCs directly stimulate growth and differentiation of B-cells and the production of antibodies. Particularly follicular dendritic cells are believed to be important in humoral immunity because they sustain viability, growth and differentiation of activated B cells. We speculate that DCs fused to tumor cells can induce an antibody-mediated immune response. Indeed, vaccinated animals that rejected the parental tumor cells displayed Meth A specific antibodies. We speculated that Meth A specific antibodies most probably belong to the IgG or IgM isotype, because after first antigenic encounter mature B cells are known to express IgM and then increasing amounts of the IgG isotype with growing affinity due to a process of isotype switching and affinity maturation. Our experiments showed that Meth A specific antibodies were of the IgG isotype and recognized unknown proteins of a molecular weight of 53 kD, 21 kD, and 16 kD. These Meth A specific antibodies were exclusively identified in tumor resistant mice but not in mice that were not in contact with cell fusion clones or were not protected from lethal tumor growth. These experiments substantially support the idea that Meth A specific antibodies are induced only after immunization with cell fusion hybrids, and that they are not present in the non-vaccinated recipient. Our next experiments will analyze these immunogenous antigens by immunoprecipitation and microsequencing. The protein sequence of these proteins might lead to the identification of new tumor antigens or it might associate known proteins to tumorigenesis. On the other hand, if the sequences correspond to that of already known tumor antigens it might serve as a proof of our results.
It is now widely accepted that the immunoglobulin-isotypes determine the effector functions of humoral immunity. IgG antibodies are closely connected to the

complement system. Complement protein binds to the Fc region of antigen-antibody complexes and triggers the classical complement pathway. IgGs can also coat antigenic particles and the bound IgGs serve to enhance the efficiency of phagocytosis by mononuclear phagocytes and granulocytes that have the ability to ingest opsonized particles as a prelude to intracellular killing and degradation. IgGs also promote the antibody dependent cell mediated cytotoxicity. Different leukocyte populations including neutrophiles, eosinophils, mononuclear phagocytes and NK cells are capable of lysing various target cells when precoated with specific IgGs. Recognition of aggregated antibodies occurs through low affinity receptors for Fcγ, called the CD16 receptor. Lysis of target cells is performed by secretion of cytokines such as TNF or IFN-γ and release of granules. A specific antibody-profile was only displayed in tumor resistant mice and therefore we speculate that these antibodies might be important for the process of tumor rejection. Additionally, antibody producing B lymphocytes are known to serve as antigen presenting cells that can provide additional support for cellular immunity.

6.14 Generation of a cellular immune response

The importance of a T cell response in antitumor immunity was demonstrated by immunizing nude mice. Athymic, nude mice that were injected with cell fusion clones died because of lethal tumor growth. Hybrid cells that were rejected in the immunocompetent animal developed to aggressive tumors and eventually killed the treated animal. Since nude mice differ from immunocompetent mice by the absence of T lymphocytes, this experiment suggest that the main reason for tumor rejection is due to the induction of cellular immunity and B lymphocytes are not the major effector cells participating in tumor rejection. Indeed, it is now well established that CTLs play a crucial role in the host's immune response to viral infections and cancer (486-488). The recognition of a tumor cell by a cytolytic $CD8^+$ T-lymphocyte depends on the endogenous processing of tumor antigens and their presentation via MHC class I complexes (489, 490). Most nuclear cells express MHC class I molecules and can therefore present tumor antigens but only APCs are coated with costimulatory molecules that are necessary for T cell activation. Thus by fusion of Meth A tumor cells with DCs the resulting fusion hybrid cell combines both the tumor antigens and the machinery that is necessary for efficient T cell activation. As a result cytotoxic lymphocytes can be activated via the biological pathways and induce a potent antitumor immune response. Indeed we could demonstrate specific activation of T lymphocytes when individuals were immunized with the cellular vaccine. Spleenic lymphocytes from the vaccinated immunocompetent animals clearly showed a proliferative response upon stimulation with fusion hybrid cells *in vitro*. Lymphocytes isolated from unvaccinated or from tumor bearing animal did not show this behavior. These cells were not preactivated by our vaccine therefore *in vitro* encounter of these cells with the cell fusion hybrids for two days is not sufficient to induce a proliferative immune response. In the next set of experiments we plan to determine whether tumor rejection is mediated by $CD4^+$ or $CD8^+$ T cells. Mice will be depleted of $CD4^+$ or $CD8^+$ cells by antibody treatment before tumor cell inoculation. Additionally the presence of Meth A specific cytolytic T cells will be demonstrated by a ^{51}Cr release assay.
However, the participation of B lymphocytes in the process of tumor rejection cannot totally be ruled out and might be a necessary part in the process of tumor rejection. Essential for B cell activation and in the induction of a humoral immune response is a complicated cross talk between B- and T lymphocytes. Helper T cells deliver contact-mediated signals to B cells and secrete cytokines that induce growth and differentiation of B cells and thereby influence the nature and magnitude of the antibody response. It is now well established that non-stimulated B cells require contact with helper T cells whereas recently stimulated B cells may be fully responsive to cytokines alone. We therefore suggest that for efficient tumor rejection

a combination of as many immune effector mechanisms as possible is necessary and only a well balanced combination of all participants will lead to a successful elimination of the tumor.

6.15 Conclusions

Our results are particularly promising because they represent a new approach to targeted immunotherapy. A fusion cell hybrid between tumor cell and antigen presenting cell offers a potential weapon against tumorigenesis, because multiple tumor antigens are combined in one cellular vaccine. Heterogeneous tumor growth and development is associated with natural selection for tumor cells that can escape therapy treatment. A tumor vaccine that can induce multiple effector mechanisms and is targeted against many different tumor antigens can produce more stringent conditions e.g. higher selective pressure on the single tumor cell. As a result one single malignant tumor cell is suddenly recognized via many different tumor antigenic properties. It cannot escape treatment just by downregulating one of its tumor antigens. The tumor cell cannot hide itself anymore and its fate is to die.

VII. Literature

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Appendix

FUSION HYBRID CLONES	B7-1	B7-2	K^D	D^D	A^D
A	- -	- -	+ -	+ -	+ -
B	+	- -	+	+	- -
C	++	++	+	+	+
D	+	- -	- -	- -	+ -
E	+ -	+ -	+	+	+
F	+	+	+	+	+
G	+	+ -	-	-	- -
H	++	- -	- -	- -	+
I	++	+ -	- -	- -	++
J	++	- -	+	+	- -
K	-	-	+ -	+ -	+ -
L	++	- -	- -	- -	- -
M	+	- -	+ -	+ -	+ -
N	++	+ -	+ -	+ -	+ -
O	- -	- -	++	++	- -
X1	- -	- -	+ -	+ -	- -
X2	++	- -	- -	++	- -
X3	++	+ -	- -	+ -	- -
X4	+ -	+ -	+ -	++	- -
X5	- -	- -	- -	++	- -
X6	+	+ -	+	+	+
X7	++	- -	+ -	++	- -
X8	++	- -	- -	+ -	- -
X9	++	- -	- -	++	- -
X10	++	++	- -	++	- -
X11	- -	- -	++	- -	- -
X12	+ -	- -	+	+ -	- -
X13	- -	- -	++	++	- -
X14	- -	- -	++	- -	- -
X15	++	- -	++	- -	- -
X16	++	- -	++	- -	- -
X17	++	+	++	++	++
X18	- -	- -	+ -	+ -	- -
X19	- -	- -	++	- -	- -
X20	+	- -	++	- -	- -

FUSION HYBRID CLONES	B7-1	B7-2	K^D	D^D	A^D
X20	+	- -	++	- -	- -
X21	++	- -	++	- -	- -
X22	++	+ -	++	++	+
X23	- -	- -	++	- -	- -
X24	- -	- -	++	- -	- -
X25	+	- -	++	- -	+ -
X26	+ -	+	+	+	- -
X27	++	+	++	+	+
X28	- -	- -	++	- -	- -
X29	++	- -	++	- -	- -
X30	+	+ -	++	+	- -
X31	- -	- -	+	+ -	+
X32	++	- -	++	- -	- -
X33	- -	- -	+ -	- -	- -
X34	- -	- -	+	- -	- -
X34	- -	+ -	++	- -	+ -
X36	+ -	- -	++	- -	- -
X37	+ -	- -	++	+	- -
X38	++	- -	++	- -	- +
X39	+	+	+	+	+
X40	+	+	++	+	+
X41	- -	- -	+ -	- -	- -

Fusions of DCs and Meth A tumor cells. Fusion hybrid clones were washed with phosphate buffered saline and stained with FITC-labeled monoclonal antibodies to mouse B7-1 (16-10A1), mouse B7-2 (GL 1), K^D (SF1-1.1), D^D (34-4-12), and PE-labeled antibodies to mouse A^D (AMS-32.1) for 30 min. on ice. Samples were washed and analyzed by flow cytometry. Expression of antigens was evaluated as high (++), good expression (+), low expression (+-), and no detectable expression (--).

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Curriculum vitae

Name:	Matjaz Humar
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Academic training:

1989-1996	Student of Technical Biology Undergraduate and graduate studies in Technical Biology University of Stuttgart, Germany
1995-1996	Diploma Student Institute for Experimental Cancer Research, Tumor Biology Center, Freiburg <u>Research project:</u> "Detection of antibodies directed against cyclin D1, cyclin E, mdm2, and erbB-2 in the serum of cancer patients."
1996	Diploma in Technical Biology University of Stuttgart, Germany
1996-current	Ph.D. Student Institute for Experimental Cancer Research, Tumor Biology Center, Freiburg <u>Research project:</u> "Induction of cellular immunity by direct pDNA transfer of a mutated p53 allele into Balb/c mice."

Scientific Lections

1. Advanced Training in FACS Analysis, *Becton Dickinson, Freiburg,* 8- 10.10.1997 (FACSComp, Lysis II) and 4-8.3.1999 (FACSCalibur System).

2. Fortbildungsveranstaltung für Projektleiter und Beauftragte für die Biologische Sicherheit (Basic Training for Group Leaders), *Albert-Ludwigs- Universität, Freiburg,* 19.09.1998.

3. Program in *Cancer Research at the Tumor Biology Center in Freiburg* - a weekly presentation and discussion of research, involving two institutes. Presentations of recent key research in *Journal Club*, presentation of own research results in *Research Meeting.*

Presentations

1. **Humar M.**, Mundt M., Groner, B.. Detection of circulating antibodies against the cellular oncoproteins cyclin E and mdm2 in the serum of cancer patients. *EUCC2 Symposium, Molecular Biology of Carcinogenesis, Strasbourg, June 9., 1995.*

2. **Humar M.,** Maurer-Gebhard M., Azemar M., Altenschmidt U., Flies B., Hermann S., Groner B.. Induction of cellular immunity by direct DNA transfer of a mutated p53 allele. *Chirurgische Universitätsklinik, Plastische Chirurgie, 1998.* Guest of Prof. Dr. G. B. Stark.

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2. **Humar M.**, Maurer-Gebhard M., Stöcklin E., Groner B.. *In vivo* administration of p53 encoding plasmid DNA efficiently protects from formation of pulmonary metastases.

3. **Humar M.**, Maurer-Gebhard M., Ricciardi-Castagnoli P., Groner B.. Transduction of dendritic cells with mutant p53 alleles creates an effective antitumoral vaccine.

4. **Humar M.**, Maurer-Gebhard M., Huber A., Flies B., Ricciardi-Castagnoli P., Groner B.. Generation of an effective cellular tumor vaccine by fusion of professional antigen presenting cells with fibrosarcoma cells.

5. **Humar M.**, Maurer-Gebhard M., Ricciardi-Castagnoli P., Groner B.. Immunization of mice with hybrid cell fusions induces B-cell activation and recognition of tumorspecific antigens in Balb/c mice.

6. **Preparation of manuscripts** in cooperation with the Chirurgische Universitätsklinik, University Hospital Freiburg, Germany.

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