Gene-based vaccines and immunotherapeutics

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DNA vaccines, comprised of plasmid DNA encoding proteins from pathogens, allergens, and tumors, are being evaluated as prophylactic vaccines and therapeutic treatments for infectious diseases, allergies, and cancer; plasmids encoding normal human proteins are likewise being tested as vaccines and treatments for autoimmune diseases. Examples of *in vivo* prophylaxis and immunotherapy, based on different types of immune responses (humoral and cellular), in a variety of disease models and under evaluation in early phase human clinical trials are presented. Viral vectors continue to show better levels of expression than those achieved by DNA plasmid vectors. We have focused our clinical efforts, at this time, on the use of recombinant viral vectors for both vaccine as well as cytokine gene transfer studies. We currently have four clinical programs in cancer immunotherapy. Two nonspecific immunotherapy programs are underway that apply adenoviral vectors for the transfer of cytokine genes into tumors *in situ*. An adenovirus-IFNγ construct (TG1042) is currently being tested in phase II clinical trials in cutaneous lymphoma. A similar construct, adenovirus-IL2 (TG1024), also injected directly into solid tumors, is currently being tested in patients with solid tumors (about one-half of which are melanoma). Encouraging results are seen in both programs. Two cancer vaccine immunotherapy programs focus on two cancer-associated antigens: human papilloma virus E6 and E7 proteins and the epithelial cancer-associated antigen MUC1. Both are encoded by a highly attenuated vaccinia virus vector [modified vaccinia Ankara (MVA)] and both are coexpressed with IL-2. Encouraging results seen in both of these programs are described.

It has been a dream of immunologists, starting with the impressive results of William Coley at the beginning of the last century (1) and reactivated in the 1970s, that the power of the immune response could be harnessed and applied to the specific elimination of cancerous cells. Cytokine molecules, which boost the immune system, first purified from tissue culture, then cloned and made available in recombinant form, have been applied to the treatment of cancer by systemic injection. This treatment has resulted in some serious dose-limiting toxicities. Nevertheless, recombinant IL-2 and IFN-α are now applied to the treatment of kidney cancer and melanoma. In this overview, we describe two cytokine gene therapy vectors that Transgene has produced and is testing clinically. These recombinant adenoviruses are injected directly into solid tumors and result in the intratumoral expression of cytokine genes in cells within the tumor. This way, high doses of cytokine are produced locally, but there is much reduced toxicity known to be associated with systemic delivery of the recombinant cytokine protein (2).

Since the 1980s (3) antigens associated with cancer cells have been identified and cloned. These cancer-associated antigens have been applied to the immunotherapy of cancer by vaccination. It has become clear that simple vaccination as applied to healthy individuals for the prevention of pathological infections rarely, if ever, works in the cancer setting. This appears to be the result of immune regulation. As part of the selective process for the growth of a tumor, the tumor itself produces a variety of immunosuppressive molecules or it reduces its own expression of antigens or antigen-presenting molecules. In addition, most tumor-associated antigens are "self" antigens and therefore are protected from immune attack by a complex immune tolerance mechanism. Nevertheless, our understanding of these mechanisms improves continually, and various cancer vaccine immunotherapy strategies are now being tested in the clinic. Below we describe two such vaccines currently in clinical development at Transgene. Both rely on the highly attenuated vaccinia pox virus, modified vaccinia Ankara (MVA) (4). One such vector, MVA-HPV-IL2 expresses the human papilloma virus (HPV)-associated oncogenes HPV16-E6 and -E7 (both mutated to maintain antigenicity but to interrupt oncogenic potential). This vector also expresses the cytokine IL-2 to provide, locally, a boost to the immune response in cancer patients whose immune system is impaired. Because the antigens E6 and E7 are viral antigens, the issue of "self-tolerance" should not pose any problems. Clinical studies with this vector are briefly described below. A second cancer vaccine immunotherapeutic, involving the epithelial cancer-associated molecule MUC1, is also described. This vector is applicable to a wide variety of common cancers. Both MUC1 and IL-2 are expressed with the intention of overcoming not only the immune anergy associated with advanced cancer, but also the self-tolerance associated with MUC1.

Overview of Results

Cytokine-Based Gene Therapy of Cancer. It is well known that a state of immune anergy or active immune suppression is often associated with cancer. Therapy with immune-stimulating cytokines is often tested but is usually associated with dose-limiting toxicities. High local cytokine production has the advantage that therapeutic doses of cytokine can be achieved at the site where it is most effective, without toxicities associated with large, systemic doses. For this approach we have constructed recombinant adenoviruses expressing genes for either IL-2 or IFN-γ with the goal of using these vectors for cytokine gene transfer to tumor cells *in situ*. We have now undertaken several clinical studies in which we evaluate cytokine gene transfer to solid tumors to generate high rates of local production of immunostimulatory cytokines using these vectors. Results from preclinical work demonstrated good cytokine expression, both in continuous cell lines and in primary human tumor models.

Adeno-IL2 has been tested in several murine tumor models and shown to induce regression of growing tumors (5). In a clinical study, adeno-IL2 (TG1024) has been injected into the tumors of 20 patients with metastatic melanoma or other advanced solid tumors. Data shows dose-dependent levels of IL-2 cytokine in the serum after repeated administrations. The

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Abbreviations: HPV, human papilloma virus; MVA, modified vaccinia Ankara; PSA, prostate-specific antigen.

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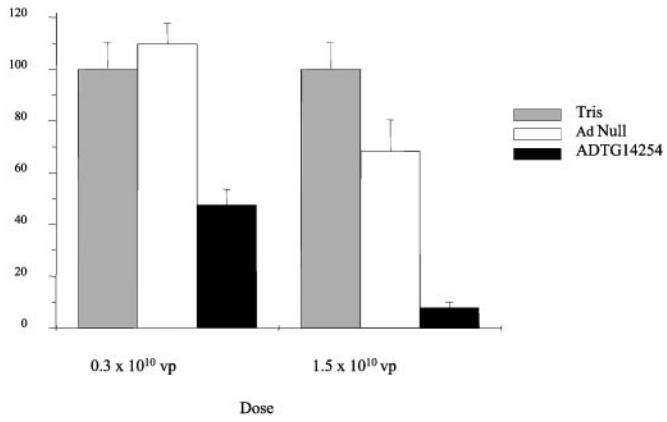

Fig. 1. Inhibition of RENCA lung metastases in B6D2 mice treated with adeno-IFNγ (ADTG14254). Six days after the i.v. injection of RENCA cells, one direct administration into the lungs with 0.3×10^{10} or 1.5×10^{10} viral particles (vp) of the ADTG14254 was accomplished by lung intubation. Lung metastases were enumerated 1 month after the treatment.

treatment was well tolerated up to the highest dose level, i.e., 3×10^{11} viral particles, with only injection site reactions and transient episodes of fever as the most commonly observed side effects. Several disease stabilizations have been observed in melanoma patients receiving the highest dose of TG1024. TG1024 is now being evaluated in combination with chemotherapy in melanoma.

Adeno-IFNγ. Similarly, an adeno-IFNγ has been constructed and tested preclinically and clinically. Because human IFN-γ is ineffective in mice, a construct, designated ADTG14254, encoding murine IFN-γ, which is otherwise identical to the adeno-human IFN-γ product TG1042, has been tested in mouse models.

In the metastatic RENCA (renal cell carcinoma) tumor model, multiple lung metastases developed when tumor cells were injected i.v. In this model, injection of AGTG14254 into the lung by intubation significantly enhanced the survival rate of animals bearing pulmonary metastases by inhibiting growth of metastatic nodules (Fig. 1).

With adeno-(human)IFNγ (TG1042), we have undertaken a phase I clinical trial as a standard dose-escalation in nine patients with advanced primary cutaneous T cell lymphomas or multilesional cutaneous B cell lymphomas. This trial has now been extended as a phase I/II trial targeting a larger patient population in different cutaneous lymphomas subtypes, with the possibility for multitumor injections. The results from 13 patients have been analyzed to date. Adeno-IFNγ was well tolerated up to the highest dose level (3×10^{11} virus particles). Only two transient serious adverse events (diarrhea and nausea/vomiting) were reported. All other adverse events were mild or moderate with injection site reactions, transient fever, and headache as the most commonly observed adverse events. Clinical responses were observed both locally (five complete and two partial responses out of 11 evaluable patients) and at distant sites (three complete responses out of nine evaluable patients), leading to an overall response rate of 60% (four complete and two partial responses out of 10 evaluable patients).

Gene transfer and expression of the IFN-γ gene on both protein and messenger RNA levels are observed, as are pronounced changes in infiltrate histological pattern, with signs of vasculitis and increases in cytotoxic immune effector cells. The detailed results of this clinical trial can be found in ref. 6. The approach of high local cytokine production within tumors, using vector-mediated gene transfer, is supported by the data from these clinical trials. Further clinical evaluation is ongoing.

Antigen-Specific Immunotherapy of Cancer. The MUC1 protein is a highly glycosylated mucin (>200 kDa), normally found at the apical surface of mucin-secreting epithelial cells in many types of tissues. MUC1 has a small transmembrane region and an intracellular tail. The bulk of the extracellular region consists essentially of a large number (20–100) of repeated segments in tandem of 20 amino acids (variable number tandem repeats). The peptide core is densely coated with oligosaccharides, conferring a rigid rod-like structure that can extend several hundred nanometers from the apical cell surface into the lumen of ducts and glands.

Cancer in secretory epithelial cells is often accompanied by excess expression of MUC1 by the tumor cells. Tumor MUC1 protein is much less glycosylated than normal MUC1 protein, revealing new peptide and carbohydrate epitopes (7) that can be specifically recognized by murine monoclonal antibodies.

Because MUC1 was identified as a cancer-associated antigen, by using monoclonal antibodies (8–10), it has been intensely studied as a candidate cancer vaccine antigen. It appears to have some unique properties in immune stimulation and recognition. Data have been published that show that MUC1-specific cytotoxic T lymphocyte activity can be detected in MUC1-immunized patients (11, 12). Non-MHC-restricted cytotoxic T cell activity has also been described (13). More recently, it has been shown that lymphocyte stimulation with MUC1 and IL-2 (+/−IL-12) can stimulate populations of natural killer T (NKT) lymphocytes that have NK activity against tumor cells and some of which have MUC1-specific cytotoxic activity (14). What is important about these cells is that the MUC1-specific cytolytic activity is independent of the MHC-I complex, which is often modified or down-regulated in tumor cells (11, 15, 16). There have also been reports of low-level MUC1 expression by activated T cells. It is intriguing to speculate that MUC1 could also play a role in the subsequent stimulation of NKT cells.

We have performed phase I studies in prostate (17) and breast cancer (12, ‡) with a vaccine vector based on replication-competent vaccinia virus, carrying genetic sequences for both MUC1 and IL-2: VV-MUC1-IL2 (TG1031). MUC1-specific T cell responses and increased NK activity in response to vaccination were observed in both phase I studies with TG1031. Some clinical responses were also observed. In the prostate study, one patient, with rising prostate-specific antigen (PSA), had his PSA drop to normal level until cessation of treatment 1 year after starting. After vaccination was stopped, PSA rose again. Upon retreatment, the patient's PSA remained stable for an additional year. PSA levels stabilized for at least 300 days in two additional patients (17). In a phase II study of TG1031 in 31 patients with breast cancer, two partial responses were observed, one of them in a patient with liver metastases (18).

The vaccine TG1031, based on replication-competent vaccinia virus, suffered some regulatory issues. Patients in France were required to be isolated in a specialized hospital facility for ≈1 week, until two consecutive PCR evaluations of blood, sputum, urine, and feces showed no evidence of viral dissemination. Nevertheless, the PCR and tissue culture evaluation of ≈200 samples of blood, urine, feces, and nasopharyngeal swabs showed no evidence of viral dissemination.

A safer and potentially more effective new vaccinia virus, TG4010, was therefore developed. The recombinant vaccinia vector contained in TG4010 is based on MVA, a nonpropagative,

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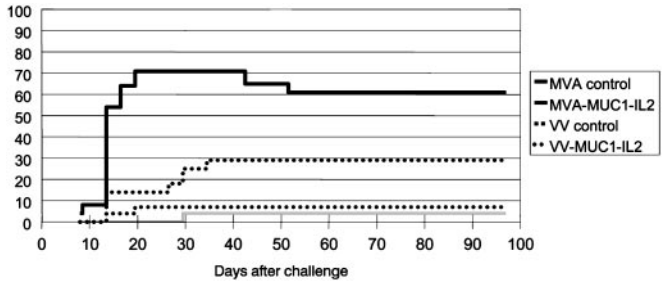

Fig. 2. Immunoprotection experiment in C57BL/6 mice inoculated with the tumor cell line RMA-MUC1 and bearing MUC1-positive tumors. Animals were first immunized three times, s.c., with vaccinia viruses expressing MUC1. One million tumor cells were injected s.c., and tumor growth was assessed.

highly attenuated vaccinia virus strain that was specially developed for immunizing high-risk patients (e.g., nervous system disorder, allergy, or skin disease) against smallpox (4). Although retaining immunogenicity, MVA has lost its ability to replicate in most mammalian cells. MVA was successfully tested without significant side effects in a variety of animal species. Moreover, the cytoplasmic location of MVA-based vectors prevents any risk of integration into the host cell genome. In humans, MVA was administered to 150,000 patients in Germany in the course of a vaccination campaign in the early 1970s. These administrations of MVA as a smallpox vaccine established the safety of the vector, including its remarkable side-effects profile.

Expression of the MUC1 sequence has been improved in MVA-MUC1-IL2 by the stabilization of the tandem-repeat portion and by putting MUC1 under the control of the stronger promoter. Like VV-MUC1-IL2 (TG1031), MVA-MUC1-IL2 contains a second sequence coding for human IL-2, a cytokine playing an important role in immune stimulation that serves as an additional adjuvant for anti-MUC1 immune responses. Animal experiments show that the MVA-MUC1-IL2 vaccine is at least as effective as VV-MUC1-IL2 in preventing growth of MUC1-expressing tumors in mice (Fig. 2). MVA-MUC1-IL2 has also been shown to be effective as a therapeutic agent in the elimination of established, growing, MUC1-expressing tumors in mice (Fig. 3). TG4010 (clinical MVA-MUC1-IL2) has shown an excellent safety profile in phase I testing and was shown to stimulate MUC1-specific T cell responses (19). It is now being tested in four phase II studies, and tolerance, in >150 patients, continues to be good. Preliminary results from the four phase II studies are as follows.

Breast Cancer. In advanced, metastatic breast cancer, TG4010 was tested as a single agent at two doses. Some disease stabilizations were observed, but no objective clinical responses were attained at either dose. The study has now been terminated.

Lung Cancer. In a second study, in advanced (stage IIIb or stage IV) lung cancer, TG4010 is being tested alone or in combination with standard chemotherapy. The trial was designed to include up to 66 patients with no prior treatment for their advanced disease. The trial is being conducted in two randomized, parallel single arms to achieve similar patient characteristics in each arm. The primary end point of the trial is tumor response rate. The patients in the first arm are being treated with TG4010 in combination with a standard chemotherapy (vinorelbine/cisplatin). The patients in the second arm are treated first with TG4010 alone for 6 weeks and then with TG4010 in combination with chemotherapy.

A classical two-stage design is being used to evaluate the tumor response rates and to assess whether the treatment has sufficient activity against the disease to warrant further development. The statistical hypothesis reflecting the chosen lower and upper target response rates to be reached (20% and 40%, respectively) requires at least five responses from 18 patients in the first stage in either arm to proceed to the second stage, and 11 responses from 33 patients at the end of the second stage.

To date, five partial responses validated by central review according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (international CT scan evaluation) have been documented in the first arm. These responses have been observed among the first 12 patients evaluated. These promising responses have justified moving forward to the second stage of the trial. The response duration ranged from 114 to 195 days, encouraging when one considers that the sample group comprised, predominantly, stage IV patients. Moreover, four disease stabilizations have also been observed.

In the second arm, treatment with TG4010 alone resulted in four disease stabilizations (91–236 days) for 4 of 16 patients. The study is ongoing, but recruitment has been completed.

Prostate Cancer. The prostate cancer trial is one of evaluating TG4010 in patients who have had primary treatment by surgery or radiation and subsequently had progressive elevation of their PSA level without documented evidence of metastatic disease,

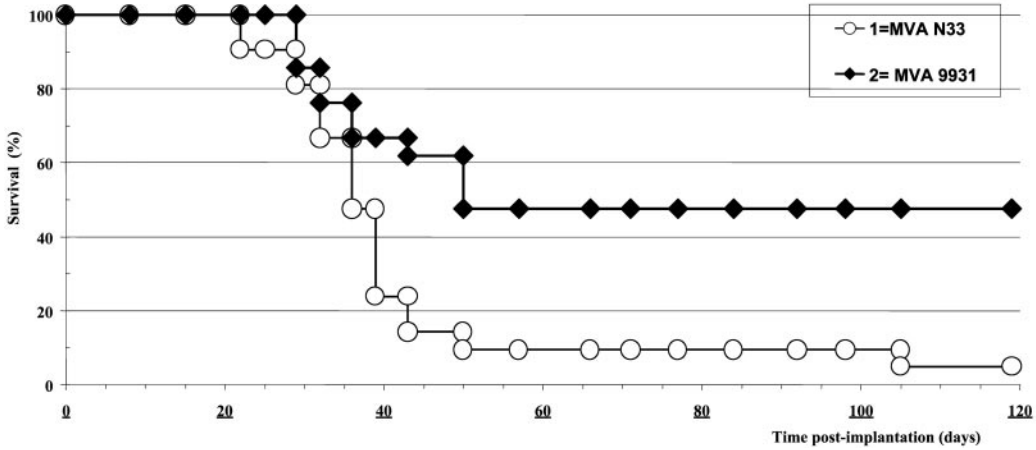

Fig. 3. Immunotherapy of growing RENC-MUC1 tumors in C57BL/6 mice. Mice were injected s.c. with 10^5 RENCA-MUC1 cells. When tumors were palpable, mice were injected s.c. with 5×10^7 plaque-forming units (pfu) of MVA-MUC1-IL2 (MVA 9931) or control MVA (MVA 33) on days 3, 10, and 17.

which suggests residual or recurrent prostate cancer. This is a phase II, multicenter, randomized, open-label trial to assess the clinical and biological effects of two different vaccination schedules. The patients in the first arm receive a weekly 10^8-plaque-forming unit (pfu) injection of MVA-MUC1-IL2 for 6 weeks and thereafter every 3 weeks. Patients in the second arm receive the same treatment every 3 weeks.

The primary efficacy end point of the trial is a decrease of 50% or more in PSA compared with the baseline level. The trial follows a two-stage design: 15 patients are treated in the first stage in each arm, with an additional cohort of 10 patients to be enrolled if at least one objective response is observed in the first stage. A secondary end point is an impact on the PSA progression rate.

Although the primary end point has not yet been reached, interim analysis performed on the first 29 patients enrolled demonstrated a statistically significant decrease in the PSA progression rate, when comparing pretreatment with posttreatment PSA values. This lengthening of PSA doubling time is statistically significant ($P < 0.0001$). The increase in PSA doubling time is, on average, 3- to 4-fold, which could translate into a clinical benefit, because the patients included in this study have pretreatment PSA doubling times of 10 months or less, placing them in a group at high risk for development of metastatic disease.

Considering that PSA doubling time is an important predictor of disease progression, the effect observed in the patients treated with TG4010 could provide a new therapeutic opportunity by delaying the administration of the conventional secondary treatments, which are known to have distressing side effects.

Kidney Cancer. In this phase II trial, patients with metastatic renal cell carcinoma, previously treated with radical or partial surgery, received TG4010 alone for 12 weeks and were then evaluated. If an objective response or disease stabilization is observed, the MVA-MUC1-IL2 monotherapy is continued. Otherwise, patients receive TG4010 in combination with a standard immunotherapy regimen (recombinant IL-2 and IFN-α).

Enrolment in this 36-patient study proceeded quickly and is now complete. Nine of 21 evaluable patients have stable disease and are continuing their treatment with MVA-MUC1-IL2 monotherapy. Further data will be available in the third quarter of 2004.

These preliminary data suggest the encouraging potential for this product candidate in metastatic renal cell carcinoma. The standard immunotherapy for this disease, characterized by an important unmet medical need, induces objective responses in $\approx$15–20% of patients and a 5-year survival rate of $\approx$10%.

HPV-Associated Cancers. HPVs have been associated with a variety of epithelial proliferative diseases, including cutaneous warts, anogenital condylomas, and epithelial cancers of the cervix, penis, and anus. The pathogenesis of cervical neoplasm follows a natural history characterized by HPV infection, a long latency period, and progression in a fraction of patients through dysplasia and carcinoma *in situ* to invasive cancer and metastatic disease. Only a few viral strains are specifically responsible for cervical neoplasms, of which HPV16 accounts for more than one-half of reported cases.

Approximately 500,000 new cases of cervical cancer are observed each year, worldwide. Cervical cancer remains an important cause of death for women in many economically underprivileged countries. Incidence and death are particularly high in Latin America and in some countries of Eastern Europe and Asia, where cervical cancer represents the second most common cancer among women.

Cervical carcinoma is usually preceded by precancerous changes (cervical intraepithelial neoplasia, or CIN) that are graded CIN 1 through 3 to denote lesions of increasing severity. The development of invasive carcinoma from CIN3 lesions occurs in one- to two-thirds of cases with a transit time ranging from 10 to 15 years. Although morbidity and mortality associated with cervical cancer have been significantly reduced, primarily because of improved diagnosis, screening of early lesions may eventually not be done, or cervical cancer might be diagnosed at a stage when lesions are inoperable and radiotherapy is the only treatment available.

Another HPV16-induced disease, vulvar intraepithelial neoplasia (VIN), although less frequent than CIN and cervical cancer, is affecting an increasing number of women. Vulvar lesions consist of either multiple, small, symmetric and pigmented papules or extending and joining lesions of the external genitalia of young women. Those lesions are graded VIN 1, 2, and 3, with the latter indicating furthest progression toward a cancerous stage. Although the evolution of the VIN is usually benign, current therapies are not satisfactory, primarily because of pain associated with the local treatment (e.g., surgical excision, electrocoagulation, laser treatment, etc.) and high rates of recurrence.

All HPV-related disorders represent an attractive target for therapies up-regulating immune response. Estimations give 10% of sexually active adults aged 15–49 infected with HPV, with only 1% displaying condyloma acuminata and $\approx$20–30% of patients with condyloma experiencing spontaneous regression. These data suggest that most people develop appropriate immunity to control viral infection, whereas others may benefit from therapies stimulating their immune system. It is in this context that TG4001 was developed for the treatment of pathologies related to HPV16 infection.

The onset of HPV-induced neoplasia involves the interaction of E6 and E7 early gene products with the proteins encoded by the tumor suppressor genes *p53* and *Rb*, respectively. To increase the antigenic response against HPV infection, Transgene developed TG4001, a frozen suspension of MVATG8042 recombinant vector particles, which harbor nucleotide sequences encoding modified HPV16-E6 and -E7 antigens and human IL-2.

MVATG8042 is based on MVA. The cognate nucleotide sequences of the E6 and E7 proteins were modified before placing them into MVATG8042, by removal of sequences encoding interfaces in contact with *p53* or *pRb*. Furthermore, to improve the immunogenicity of modified E6 and E7 proteins, the respective genes were fused to heterologous sequences encoding secretion signal and membrane-anchoring domains.

Preclinical evaluation has been carried out in BALB/c mice by using the TC1 tumor model (20). This vaccine has been tested in three phase I clinical studies and was shown to be safe and well tolerated. The MVA-HPV-IL2 cancer vaccine candidate is now being evaluated in three phase II clinical trials, at different stages of HPV-related diseases and according to different doses and treatment modalities. Results of phase II trials are as follows.

CIN. Encouraging results have been observed at the highest dose administered, including clinical and histological improvement associated with virology clearance. This improvement was not reported at the lowest dose, where patients were identically managed. A few patients showed signs of early efficacy as early as week 6.

VIN. The recruitment into this study has been stopped. Analysis of the data does not show significant efficacy in the MVA-HPV-IL2 treatment arm, compared with the control arm (placebo).

Cervical Cancer. Two patients had stable disease at month 6 or later (primary efficacy criteria) that did not reach the efficacy threshold foreseen per protocol. The protocol was amended to

allow patients to receive chemotherapy after disease progression during the study: two partial responses and two disease stabilizations have been reported, suggesting a potential synergy between immunotherapy and chemotherapy in this indication, similar to the results from the study with MVA-MUC1-IL2 in lung cancer.

Discussion

Cancer immunotherapy vectors are now being tested in phase I and phase II clinical trials at Transgene. The choice of clinical setting is of utmost importance for products such as these, to prove their effectiveness, because most studies must be carried out in late-stage cancer patients. It is well know that patients in this group are usually in a state of immune anergy and so are less likely than early-stage patients to mount a vigorous immune response. Nevertheless, some very encouraging results have been observed with each of the vectors described.

Knowledge of how cancer-associated immune anergy and self-tolerance are maintained continues to accumulate. The application of strategies that involve CTLA-4 blockade are being applied clinically (21). In addition, blockade of molecules, such as GITR, by which the newly identified T regulatory cells function, may also provide systems for overcoming immune anergy and self-tolerance (22). We have described four cancer immunotherapy vectors that look to have a future in cancer immunotherapy.

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