Cancer Immunol Immunother (2004) 53: 204–216
DOI 10.1007/s00262-003-0483-7

REVIEW

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Immunological inhibition of carcinogenesis

Received: 12 September 2003 / Accepted: 24 October 2003 / Published online: 13 January 2004

Abstract The combination of new information provided by fundamental immunology, along with the refinement of genetic engineering techniques has given scientists the capacity to produce vaccines able to inhibit the growth of most if not every transplantable tumor. However, when faced with already established tumors, vaccines fail to afford any significant protection. Many studies are underway which seek to overcome this gloomy situation. However, another possibility is to follow the indications provided by a large quantity of experimental data and to evaluate the possibility of using immunotherapy to prevent the initial stages of tumor growth. Is it possible to prevent an autologous tumor by means of a vaccination performed before tumor onset? Could antitumor vaccines be a new form of preventive medicine in the wake of Jenner, Pasteur, and other pioneers? In this paper it is our intention to review the results obtained by our laboratory in the attempt to use natural and adaptive immunity in the control of carcinogenesis. Natural immunity boosted by IL-12 and IL-2 significantly hampers the progression of mammary lesions occurring in HER-2/*neu* transgenic mice genetically predestined to develop lethal mammary carcinomas. Specific immunity elicited by DNA vaccination provides a much stronger inhibition of the development of mammary lesions, and a significant number of transgenic mice are tumor free at 1 year of age. These experimental data suggest the possibility of using immunity as a means of controlling preneoplastic lesions and protecting healthy persons at risk of developing cancer.

This work was presented at the first Cancer Immunology and Immunotherapy Summer School, 8–13 September 2003, Ionian Village, Bartholomeio, Peloponnese, Greece.

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Keywords Tumor prevention · Mammary carcinogenesis · Cytokines · DNA-vaccination · HER-2/*neu*

From immune surveillance to tumor immunoediting and clonal escape

The immune system is a remarkably adaptive defense that evolved in vertebrates to protect them from invading microorganisms. By contrast, it plays an ambiguous and changeable role in the protection against cancer. Immune reactions can be of critical importance in either enhancing tumor oncogenic ability [19] or impairing it [44]. Yet, in the past century, Ehlrich and co-workers [2] observed the presence of infiltrates of mononuclear cells around or inside the tumor mass. This finding led them to suppose that tumors could be recognized and inhibited by the immune system. A more articulate interpretation of the protective role of the immune system was then provided by the theory of immune surveillance progressively elaborated by Lewis Thomas and Macfarlane Burnet. The central tenet of this theory is that "the immune system protects the host from cancer by detecting and destroying newly-formed neoplastic cells" [44]. This immune surveillance theory, including its critics and the various experiments supporting and disproving it, has shaped the last 40 years of research in tumor immunology.

The result of this research is that some crucial points originally predicted by the theory are now firmly established. Firstly, human neoplastic cells do express tumor-associated antigens (TAAs) in a way that immunologically differentiates them from normal cells, and secondly the immune system can be activated to effectively react against tumor cells [7]. While these and a few other facets of antitumor immunity are well defined, it is still difficult to assess the result of the influence of the immune system on the natural onset of a tumor. In several circumstances, the role of inflammatory reactions

on the proliferation of incipient tumors can be clearly assessed [2, 19]. These data now offer fresh molecular interpretations of the "old" observations concerning the enhancement of tumor onset due to immune reactions, and suggest that the tumor may be generated by cytokines and antibodies as a result of a weak antitumor immune response. On the other hand, the increased tumor incidence associated with congenital and acquired human immunodeficiencies, as well as with experimental immunodeficiencies in mice, highlights the importance of immunosurveillance on tumor growth. Here too, new data are providing molecular interpretations [23]. Indeed, the advent of targeted gene mutations in mice has led to a revolution in our capacity to study the immunosurveillance of spontaneous tumors. Several laboratories are monitoring spontaneous tumor development in various gene-targeted mice. Mice that lack perforin, T- and B-cell immunity ($RAG2^{-/-}$), interferon γ receptor ($IFN\text{-}\gamma R1^{-/-}$), or signal transduction molecules involved in immune responses ($STAT1^{-/-}$), are more prone to spontaneous and induced carcinogenesis than normal mice [23, 45]. This new knowledge has reinvigorated the idea that the immune system can constantly protect the host from tumor initiation and progression. Unfortunately, the results of this immune surveillance are often poor or null due to the genetic instability of the tumors. Paradoxically, the final result of this "cancer immunoediting" [23] and selection of immunologically invisible tumors [25] can be a tumor actually fitter to continue growing in immunocompetent individuals. When surveillance fails to remove all tumor cells, it "remodels" the incipient tumor, which is then able to escape and expand.

The concept of "immunoediting" and the selection of clones able to escape the immune reaction not only relates to the control of incipient tumors. It has also led to an understanding of one of the most substantial reasons for the failure of vaccines used to "cure" established tumors. Only sporadic, transient tumor regressions and extended survival are commonly observed [7]. Having evaded innate surveillance mechanisms and established a tolerant environment, established tumors can progress by turning their genetic instability to advantage to counter adaptive immune responses in various ways. Vaccines administered to a cancer patient must thus contend with a wide gamut of evasion strategies, some general and others peculiar to each tumor [15]. However, genetic instability is the ace up a tumor's sleeve [16]. The pressure elicited by specific immunity favors the selection of clones that no longer express the antigen targeted by the immune attack, nor the histocompatibility glycoproteins required for recognition by T lymphocytes. An established tumor can be seen as an aggregate of millions of genetically different and unstable cells, each endowed with clonogenic potential. The time frame needed to establish immune defenses through vaccination builds the perfect conditions for the immune selection of clones invisible to T lymphocytes. After an initial shrinkage due to the killing of tumor cells expressing the antigen, the immune attack may well be followed by the progression of a more anaplastic tumor with a higher proliferative and metastatic potential [14]. An active "immunoediting" makes the recurrent tumor free of target antigens (Fig. 1).

How to study the immunological prevention of tumors

Most of what it is known about the possibility of using immunity to inhibit tumor growth comes from experiments in which tumors are transplanted in preimmunized healthy syngeneic rodents. Even tumors that in more conventional experiments were unable to induce a protective immune response are promptly rejected when the syngeneic recipients are preimmunized with vaccines able to trigger an appropriate response [10, 29]. Overwhelming evidence shows that the eradication of these fast-growing tumors rests on T-cell reactivity, while B cells may interfere with the efficiency of the reaction [38]. The error intrinsically inherent in experiments of this type is the use of fast mouse models to study a chronic disease.

By contrast, whether activated immunity can prevent the slow progression of an early neoplastic lesion is a topic rarely addressed in experimental systems [36]. The importance of immune mechanisms capable of persistently preventing the progression of initial neoplastic lesions and leading to their eradication is still largely undetermined. This assessment is important since it may provide new information not entirely coincidental with that obtained studying transplantable tumors [39]. The issue is to study models of cancer that will provide indications that can be extrapolated to clinics, by approximating as best as possible human oncogenesis [45].

One possible way to assess the potential of immunity in the prevention of tumorigenesis is to use genetically modified mice that develop a tumor as a consequence of mutation and overexpression of defined oncogenes, as well as of the loss of function of onco-suppressor genes

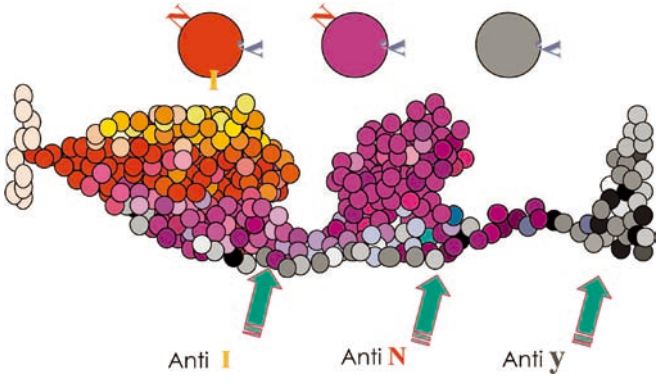

Fig. 1 Genetic instability and clonal heterogeneity limit the curative potential of a vaccine. Therapeutic vaccination deals with a typically Darwinian selection and competition between the distinct clones and the elicited immune response

or their knockout. In these mice, tumors become evident after a period characterized by progressive stages of tumorigenesis while the relationship between the tumor and the surrounding tissues is preserved [15]. The ability to reproduce the development of autologous tumors, the occurrence of invasion and metastasis, and the presence of an intact immune system are other key elements of these genetically modified mice. Despite these important analogies with the human situation, these mouse models of cancer are not devoid of pitfalls. The genetic alteration may not follow the same developmental expression pattern as the genetic alteration in humans, because of the peculiarities of the transgene promoter, and the pathogenic mechanisms may not necessarily be superimposable on those involved in the pathogenesis of human natural tumors. The timing of the first expression of the oncogene also has crucial immunological importance since it may directly determine the intensity of immune tolerance.

While there are several models of mice with gene alterations that predispose them to cancer, some lack practical utility since the tumor arises after a long period of latency, or in a small percentage of mice only, or only in particular conditions such as pregnancy. In some models the tumor appears as the result of a gene alteration that has no correspondence to human pathology. Therefore the use of a genetic model of cancer is a compromise between the features of the model, its handiness, and the specific problem that it permits one to address.

BALB/c mice transgenic for the rat HER-2/*neu* (BALB-neuT): a model for breast cancer

Nearly a hundred transgenic models of spontaneous mammary cancer have been developed, and a detailed pathological description has been provided [12]. Of these, mice that develop mammary tumors as a consequence of an alteration of the HER-2 oncogene appeared to us to be an interesting model for studying the influence of natural and adaptative immunity on HER-2/*neu* (ErbB-2 in human) carcinogenesis. The HER-2/*neu* oncogene codes for a transmembrane protein (p185neu) that is a tyrosine kinase growth factor receptor, homologous to other members of the epidermal growth factor (EGF) receptor family. Overexpression or mutation of p185neu favors the formation of homodimers or heterodimers with other EGF receptors, which transduce constitutive growth signals in a ligand-independent way. While a lot of information is now available on human, mouse and rat HER-2/*neu*, the rat HER-2/*neu* (rHER-2/*neu*) was the first to be identified and studied and is still the most widely examined [43]. In the rat there are two forms of this oncogene. The wild type form is also defined as the nontransforming (rHER-2/neuN) oncogene and promotes tumor growth only when it is overexpressed on the cell membrane. By contrast, its transforming or activated form (rHER-2/neuT) displays a single point mutation at position 664 in the transmembrane domain (TM) that leads to the replacement of a valine by a glutamic acid residue. On the cell membrane, the negative charge of the glutamic acid heads toward the formation of a H-bond with an alanine at position 661 of another p185neu molecule as well as with other receptors of the EGF receptor family [3]. These homodimers and heterodimers spontaneously transduce proliferative signals responsible for the neoplastic behavior of the cell [27]. In human cancer, there is no such mutation, but only an amplification of the HER-2/*neu* gene copy number and an overexpression of p185neu on the cell membrane. ErbB-2 is overexpressed in about 30% of breast cancer patients. However, recent studies led to the discovery of human splice variants of HER-2/*neu* that cause conformational modifications functionally similar to those introduced by mutation [42].

Thanks to the work of Leder and Muller [34], there are several lines of transgenic mice that start to overexpress the rHER-2/neuN and rHER-2/neuT at a distinct period of their lives, and which eventually develop HER-2/*neu* mammary carcinomas. One of the most aggressive models of HER-2/*neu* mammary carcinogenesis is that of our BALB-neuT mice transgenic for the rHER-2/neuT oncogene under the transcriptional control of a long terminal repeat sequence from a mammary tumor virus. We obtained these mice starting from a non-inbred HER-2/*neu* transgenic mouse after approximately 3 years of backcrossing with BALB/c mice [9]. Virgin female BALB-NeuT mice develop mammary carcinomas at a high multiplicity (all mammary glands are affected) and a relatively short latency (about 4 months). In 3-week-old BALB-NeuT mice, rat p185neu is markedly overexpressed on the surface of the cells of the rudimental mammary gland. At 6 weeks, the rat p185neu cells give rise to a widespread atypical hyperplasia of small lobular ducts and lobules. Foci of ''in situ'' carcinoma first apparent around the 15th week evolve into invasive lobular carcinomas by the 20th week. Ten weeks later invasive lobular carcinoma is present in all the glands [22].

BALB-neuT mice may not appear to be a perfect model for studying the immune prevention of HER-2/*neu* tumors since the transgene rHER-2/*neu* codes for a xenogenic rat p185neu. However, rat p185neu shares 94.8% sequence homology with mouse p185neu [16]. Moreover, in transgenic mice p185neu is expressed in the thymic stroma of newborn transgenic mice [15], and we demonstrated that it is markedly and diffusely overexpressed in multiple epithelial tissues during weaning. This early overexpression of rat p185neu along with the absence of any cellular and humoral reactivity against the tissue cells overexpressing it [15] gives rise to a significant challenge to the induction of protective immunity. While in wild-type BALB/c mice various forms of vaccination elicit strong cellular and humoral immunity and confer full protection against challenge by rat p185neu tumor cells, the same vaccines fail to elicit the

strong and rapid reactivity of high avidity T cells required for the eradication of transplantable tumor cells in BALB-neuT mice [20, 40]. In fact, the pattern of expression of rat p185neu in BALB-neuT mice and the adult tolerance elicited mimic a few features of the "adult" tolerance that patients display toward several TAAs.

Breast cancer is the most frequent malignancy in women worldwide. In many communities, early diagnosis programs and genetic screening lead to the identification of an increasing number of women bearing small initial carcinomas, preneoplastic lesions, or simply at high risk of developing a mammary tumor. In many instances the therapeutic options offered to potential patients are crude. These considerations prompt one to assess the potential of distinct procedures to halt carcinogenesis and cure preneoplastic lesions.

The significant period of time required for achieving meaningful protection and the possibility that a definitive cure might be impossible to find form the major drawbacks in studying BALB-neuT mice. At the same time, however, therein lies the value of this model, since complete remissions are just as elusive here as they are in humans. In fact, BALB-neuT mice provide a "hard" model of mammary carcinogenesis since they are genetically predestined to develop lethal invasive carcinomas in all ten mammary glands by the 21st week of age. Moreover, since the transforming oncogene is embedded in the genome of these mice the induction of an immune response cannot eradicate the lesion and definitively protect the mice. On the contrary, the induced immune response is engaged in a long-lasting confrontation between the inborn oncogenic rHER-2/*neu* signals and the sustained inhibitory potential of the immune reactions. To be of significance, each experiment with BALB-neuT mice needs to be very long as compared to conventional immunization-challenge experiments. In most cases experiments last 1 year. This unusual length makes the study of immunological functions difficult since it is not easy to decide when it is appropriate to test them. Long experiments are demanding and expensive. The reward is that these experiments somewhat approximate the human situation where the tumor-host relationship is a long-lasting and dynamic event, and not a 2- to 3-week affair.

Stimulation of innate immunity in cancer prevention

Numerous data obtained in patients as well as in mouse models of cancer have shown that proinflammatory cytokines such as IL-2, IL-12, and interferons elicit so strong a modulation of natural immune reactivity that it can result in tumor rejection. The mechanisms leading to tumor shrinkage vary according to the function of the cytokine, its dose, and the method of administration. The fascinating issue is that modulation of the natural immune response along with the stimulation of antiangiogenesis can be applied to a broad range of persons at risk: individuals with a genetic risk of cancer, individuals exposed to high doses of carcinogens, patients with a preneoplastic lesion, and those who probably have a minimal residual disease following conventional treatment. Nonspecific immunomodulation can be applied irrespective of the antigenic profile of a patient's foreseeable tumors. The slow but consistent progression of mammary carcinogenesis in BALB-neuT mice was immediately seen as the ideal situation for assessing the power of cytokine-activated natural immunity at distinct stages of the tumorigenic process.

Systemic recombinant IL-12

IL-12 is one of the most promising cytokines with the potential to activate an effective immunological response to cancer. Firstly, it has strong inflammatory properties and causes the induction of other proinflammatory cytokines, degranulation of neutrophils, the formation of lipid mediators, and activation of the coagulative and fibrinolytic systems that together can provide an environment of multiple danger signals for tumor antigens, suitable for the activation of professional antigen-presenting cells (APCs) [48]. IL-12 directly and indirectly activates innate immune effector cells such as neutrophils and NK cells and promotes their secretion of substances that alter the tumor microenvironment and stimulate expression of adhesion molecules that mediate trafficking and homing of APCs and specific immune cells [14]. Together with these immune-regulatory functions, the exceptionally long elimination half-life of IL-12 in comparison with other cytokines, forms the rationale for use of recombinant IL-12 as an anticancer agent (Fig. 2).

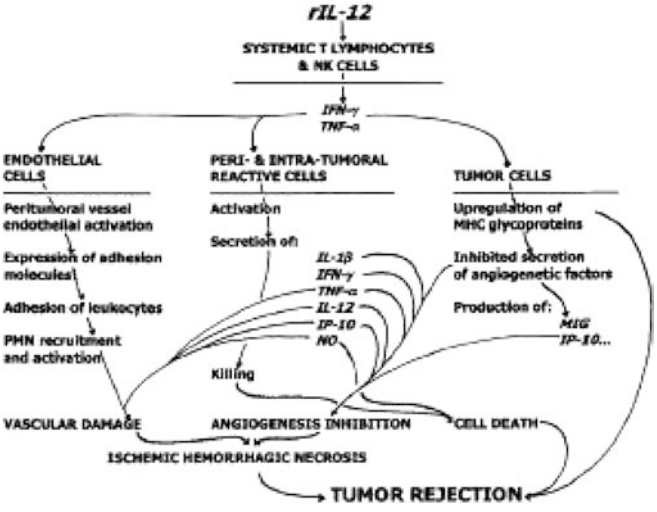

Fig. 2 Interpretation of a few of the main events leading to rIL-12–provoked tumor inhibition in vivo. The three major cell populations involved are underlined. A few of the factors whose activity has been established are italicized. *Arrows* suggest causal and temporal relationships (Reproduced with permission from [15])

In vivo protection

During our attempts to cure incipient transplantable tumors, we have observed that the natural reactions boosted by systemic administration of IL-12 lead to the regression of the great majority of these 7-day-old tumors [14, 15]. While IL-12 was injected systemically, a complex immunological reaction was evident at the tumor site. A profound inhibition of tumor angiogenesis and the damage of tumor-associated blood vessels were central issues [8, 15].

To evaluate the potential of IL-12 in the inhibition of progressive phases of mammary carcinogenesis, 100-ng IL-12 (Genetics Institute, Cambridge, MA) was injected intraperitoneally daily for 5 days/week, 1 week on, 3 weeks off, in transgenic BALB-neuT females. This ''chronic'' IL-12 administration started in the 2nd week and continued until the 25th week. Both a delay in the onset of the first tumor, and a 50% reduction in the number of mammary glands with a palpable tumor at 33 weeks when the experiment ended, were observed [8, 9]. We also set out to define the progression stage at which the IL-12–induced reaction is most effective. Should IL-12 administration be proposed as a preventive measure in potential patients only, or can it also be of benefit once overt preneoplastic lesions have been diagnosed? This is a significant question since genetic screening programs are singling out healthy potential patients, and early diagnosis programs are detecting preneoplastic lesions [24]. Groups of BALB-neuT females received the same overall dose of IL-12, but the treatment was begun at different times during carcinogenesis. To assess whether IL-12 was also effective during later phases, groups of mice were first treated at the 13th week of age, when hyperplasia takes the form of a carcinoma in situ. Courses continued until the 25th week. This ''late'' treatment did not delay the onset of the first tumor, but nonetheless reduced the number of tumors at week 33 by 22%. Other mice received an ''early'' treatment begun in the 2nd week and continued until week 14. In these mice, the delay of first tumor onset and the reduction in the number of tumors were significantly higher than in the ''chronic'' treatment. When the ''early'' treatment was further split into shorter 4-week administration schedules, much less protection was observed [9].

In addition to timing, the dose of IL-12 is another critical issue. The above experiments showed that inhibition was achieved with 5-day injections of 100-ng IL-12, whereas doses 10 and 50 times lower were almost ineffective. Extrapolation of these findings to a clinical setting suggested that IL-12 treatment could be a sensible approach for healthy women with a genetic risk of cancer, though it would be very poorly effective in patients with preneoplastic lesions. Moreover, an equivalent of the total dose of IL-12 / body weight and the heavy schedule of administration would hinder the use of IL-12 in the prevention of human mammary tumors.

Therefore, we explored whether a much lower dose of IL-12 administered when adult mice already present full-blown atypical mammary hyperplasia is as efficacious as much earlier and heavier treatments [17]. Carcinogenesis was significantly hampered in BALB-neuT mice receiving 16 intraperitoneal administrations of 100-ng IL-12 divided into four courses of a single weekly injection for 4 weeks followed by a 3-week rest. Both a delay in the onset of the first mammary tumor and a reduction in the number of mammary glands with a palpable tumor at 33 weeks were found. All IL-12–treated mice were free of palpable tumors at 20 weeks, when more than 50% of control mice already displayed palpable tumors. At week 24, all control mice displayed tumors while 76% of the treated mice were still completely tumor free. The number of tumors per mouse was also significantly lower in the IL-12–treated mice. To asses if all four courses of IL-12 treatment were necessary for effective tumor inhibition, in another set of experiments BALB-neuT mice received only the first two or three courses of IL-12 [17]. While three courses were still effective, though to a lesser extent, two courses delayed the onset of the first mammary tumor, but all glands had a palpable tumor at 33 weeks. These data indicate that inhibition of carcinogenesis following weekly IL-12 injections is no less marked than that previously observed with five injections per week [8, 9, 17].

In conclusion, early IL-12 administration would seem unnecessary, since the present findings show that it is still very effective if commenced when widespread atypical hyperplasia is already evident in all ten mammary glands. In the human setting, it might thus be possible to start IL-12 administration when an overt preneoplastic lesion is evident and not confine it to healthy persons with a genetic risk. Moreover, the total dose of IL-12 injected and the frequency of these administrations can be greatly reduced from the levels used in previous studies, with no loss of efficacy [17].

Cellular and molecular basis of the antitumor effect of IL-12

The ability of systemic IL-12 to selectively activate a local reaction at the tumor site or where carcinogenesis advances is the most peculiar outcome of our experiments [16]. This remarkable selectivity of IL-12 is probably due to its antiangiogenic activity that is clearly evident on the fragile capillaries sprouting during tumor angiogenic switch. At this stage, endothelial cells respond best to IL-12, while late administrations had little effect [17], presumably because the mature and differentiated blood vessels of more advanced lesions, as well as normal blood vessels are much less sensitive to IL-12–induced inhibition. The antiangiogenic effect appears to directly inhibit tumor proliferation as well as the progression of carcinogenesis [46]. Immunohistochemical staining with anti-CD31 monoclonal antibody showed that in the mammary glands of BALB-neuT mice the occurrence of rich microvascularization inside preneo-

plastic lesions correlates with their progression toward carcinoma [22]. The most significant inhibition of progression of carcinogenesis is observed with the "early" treatment, when IL-12 courses administered between the 2nd to the 14th week induce both scarce vascularization and poorly developed hyperplastic foci [9]. If the effect on endothelial cells is the starting point, downstream mediators, released by endothelial cells as well as by lymphocyte recruited by IL-12–activated endothelia, trigger a complex and multicell-mediated inflammatory reaction [46]. IL-12 triggers lytic activity and mediator release from macrophages, natural killer, and T cells directly or through downstream IL-2, IFN-γ, and tumor necrosis factor α (TNF-α). Moreover, third-level mediators such as TNF-α, IL-1β, iNOS, CXCL9, and CXCL10 may well be responsible for vessel wall injury, and the activation of infiltrating leukocytes able to counteract the continuous generation of transformed cells. IL-12 efficacy probably rests on the sum of these activities, and not simply on the blocking of tumor angiogenesis, important as this may be [8].

IL-12 modulation of tumor genetic programs

Subsequent studies showed that IL-12 antitumor activity is even more evident, since IL-12–elicited IFN-γ induces overexpression of MHC glycoproteins by tumor cells and induces tumor cells to generate antiangiogenic activity. Distinct lines of tumor cells change gene and protein expression when cocultured with T lymphocytes activated in the presence of IL-12 [16]. These genetic modulations suggest a new way by which the immune system affects the growth of a tumor so that it becomes an agent in its own inhibition. The ability of IL-12–activated lymphoid cells to modulate the angiogenic program of tumor cells [16] and endothelial cells [46] rests on the induction of the prolonged release of large amounts of IFN-γ and TNF-α in leukocytes. These downstream mediators act on neoplastic and endothelial cells in which they down-regulate the production of proangiogenic molecules such as VEGF and bFGF, and up-regulate the release of antiangiogenic factors [16]. Provisional unpublished data from our laboratory also indicate the ability of IL-12 to mediate the induction of CXCL11 production in both endothelial and tumor cells. The modulation of genes involved in tumor angiogenesis indicates that the interplay between soluble signals actually involves three players: lymphoid, endothelial, and tumor cells. The active role of tumor cells in mediating the antiangiogenic activities of leuckocyte-derived cytokines is also demonstrated by the inability of IFN-γ to inhibit tumor angiogenesis if tumor cells themselves are IFN-γ resistant [18].

The adjuvant effect of IL-12

A different consideration of IL-12 in anticancer treatment is to think of it not as a single therapeutic agent, but as a nonspecific modulator component for antigen-specific vaccination. Recently, IL-12 has been identified as a powerful adjuvant substance in a variety of vaccination models of infectious disease. Stimulation of IL-12 production is also considered an important working mechanism in vaccination, whereby classical adjuvant substances exert their effects. IL-12 has several characteristics that seem essential for its adjuvant effects [48]. Regarding its potential in vaccination, IL-12 activates innate immune cells and promotes production of cytokines and chemokines, thereby mediating the attraction of other innate as well as specific immune cells to the region [15]. The environment, conditioned by IL-12, is thus suitable for activation of APCs and to prevent tolerance induction toward tumor antigens. It has recently been demonstrated that IL-12 can even reverse tolerance in vivo [30].

Our data show that IL-12 is an effective adjuvant for both cellular [35] and DNA vaccines (Spadaro et al., manuscript in preparation) aimed to prevent carcinogenesis in BALB-neuT mice. In addition, the delay in carcinogenesis progression due to early administration of systemic IL-12 significantly delays the initiation of antigen-specific antitumor vaccination.

Systemic IL-2 cDNA

Among its crucial regulatory roles, IL-2 boosts the activity of NK and T cells, inducing LAK cells [26], and elicits many other reaction mechanisms that are part of natural immunity. Our previous work has shown that the local presence of IL-2 in a tumor growth area or tumor-draining lymph nodes triggers a potent and multicell-mediated antitumor reaction in both experimental models and head and neck patients [13, 21]. Nevertheless, the important side effects of IL-2 made its systemic administration a perplexing issue and the most effective route of IL-2 administration is still a subject of active investigation. In most of the studies, IL-2 has been administered as soluble protein, or as protein variously conjugated or contained in microsomes. We are evaluating the ability of an IL-2 cDNA-expressing plasmid bound to unilamellar nanovesicles composed of cationic lipids to inhibit carcinogenesis in BALB-neuT mice. These vesicles are composed of cationic lipid DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride) and cholesterol at a 1:1 ratio. Positively charged plasmid/nanoparticles (IL-2-DNA nanoparticles) were prepared at a 1:3 $-/+$ charge ratio in 10% lactose by mixing the IL-2 coding plasmid with the nanoparticles (Fig. 3) [5]. When IL-2-DNA nanoparticles are administered intravenously they may extravasate at the site of higher vessel permeability due to neoangiogenesis, namely neoformed tumor vessels. This typical feature of tumor-induced newly formed vessels permits the selective accumulation of nanoparticles at tumor sites. As a consequence, IL-2 cDNA delivered by nanoparticles into the growing tumors may

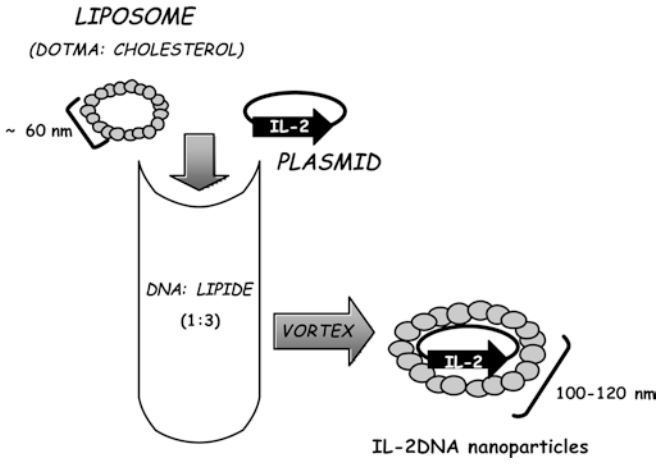

Fig. 3 Liposomes composed of cationic lipid (DOTMA) and cholesterol at a 1:1 mole ratio were prepared at a 1:3 −/+ charge ratio in 10% (w/v) lactose by mixing the IL-2 cDNA plasmid under controlled conditions

transfect tumor cells, activating an antitumor immune reaction. We have shown that the antitumor activity induced by IL-2-DNA nanoparticles was efficacious against primary tumors [6]. As a result of dose-optimization studies, we have identified administration of 30 μg of IL-2-DNA nanoparticles twice at 7-day intervals as the best dose regimen. BALB/c mice bearing established rat p185neu–positive tumors, and a cell line expressing rat p185neu, were treated with 30 μg once, 10 days posttumor injection, and again 7 days later. The treatment resulted in the complete regression of tumor in five out of nine mice.

Transgenic BALB-neuT mice that received IL-2-DNA nanoparticles at week 14 and 16 were free of palpable tumors at week 21. At 30 weeks, while a tumor was present in all glands of the controls, treated mice displayed a mean of seven glands with tumor. Moreover, in mice receiving IL-2-DNA nanoparticles the tumors grew significantly slower than in controls.

Antigen-specific immune stimulation in cancer prevention

The great preventive potential of specific immune reactivity has so far been dismissed as uninteresting since neither the type nor the antigenic makeup of a future tumor can be foreseen. This serious drawback, however, is gradually being resolved by epidemiological and genetic studies. The risk of particular types of cancer can be assessed as a function of sex, age, family history, or genetic makeup and much is known about the antigens most frequently expressed by tumors. Evaluation of safety, definition of the appropriate target antigens, and the setting of surrogate endpoints as predictors of efficacy are needed before tumor prevention vaccines can be accorded serious attention. Yet these are accessory matters. The basic question is whether a specific cancer can be prevented by immune vaccination.

Because of these considerations, we evaluated whether vaccination can inhibit the progression of HER-2/*neu* carcinogenesis in BALB-neuT mice by using cell, proteins, peptides, and DNA as antigens. To date, the most interesting data have been obtained by using DNA vaccines.

DNA vaccines

These vaccines are molecularly defined reagents that are easy to construct and which elicit long-lasting cellular and humoral responses to a variety of antigens. In recent years, clinical trials have shown that DNA vaccines are nontoxic and well tolerated, though the responses their application induces are low and vary from one individual to another. Plasmids coding for the extracellular (ECD), intracellular (ICD), and extracellular and transmembrane (ECD-TM) domains of rat p185neu were inserted in the pcDNA3 vector [39].

When BALB-neuT mice were immunized at the 6th, 12th, 18th, and 24th week of age with 100 μg of DNA plasmids coding for the ECD-TM domains (Fig. 4) injected into the quadriceps muscle, 57% of them did not display palpable masses at week 33 [39]. Plasmids coding for the ECD domain were less effective as vaccines, whereas their protective potential was increased by their association with a small nonapeptide from human IL-1β [40]. Since no significant inhibition was found in mice receiving empty plasmids or plasmids coding unrelated proteins, these findings show that preneoplastic lesions are appropriate and rational targets for specific immunological attack. Antitumor immunity is not only effective against the artificial transplant of tumor in preimmunized recipients. It is also effective when elicited in mice harboring a preneoplastic lesion that progresses as a natural consequence of a gene defect. These findings raise several interconnected questions whose answers form the goals of ongoing experiments:

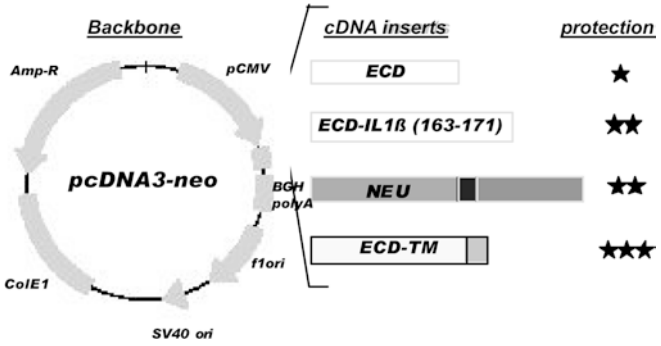

Fig. 4 BALB-neuT mice were vaccinated with different plasmids, coding for various portions of the rat p185neu protein. All cDNA were cloned into a pcDNA3 vector. The most effective plasmid was the one coding for the ECD-TM domain of rat p185neu. The plasmids coding for the full length and ECD plus IL-1β were less effective, while the ECD alone was the least effective

– How wide is the window of time in which a preneoplastic lesion can be inhibited?
– How can the efficacy of these vaccinations be prolonged?
– How can the efficacy of these vaccinations be increased?
– Which immune mechanisms are involved?
– Are these data restricted to HER-2/*neu* carcinogenesis or are other forms equally sensitive to immune attack?

While most of the experiments specifically addressing these questions are still in progress, a few glimpses of the eventual answers to these questions can already be put forward.

How wide is the window of time in which a preneoplastic lesion can be inhibited?

Our data suggest that the efficacy of vaccination decreases when its commencement is delayed, though substantial protection is still provided if it is administered when preneoplastic lesions are still undetectable.

In the first set of experiments, the protective role of DNA vaccination was assessed in BALB-neuT mice immunized when their rudimental mammary glands overexpressed rat p185neu on the surface of the cells. DNA vaccination elicits an anti-rat p185 antibody response and CTL activity, and markedly hampers the progression of carcinogenesis [11]. At 33 weeks of age, when control BALB-neuT mice display palpable tumors in all mammary glands, about 80% of immunized mice are tumor free, and tumor multiplicity is markedly reduced (Fig. 5). Tumor-free mammary glands still display the atypical hyperplasia of the early stages of carcinogenesis, and a marked down-modulation of rat p185, along with a massive reactive infiltrate. However, BALB-neuT mice protected against mammary carcinogenesis fail to efficiently reject a challenge by transplantable syngeneic tumor cells overexpressing rat p185neu on their cell membranes. This suggests that the mechanisms required for the rejection of transplantable tumors may not coincide with those that inhibit the slow progression of carcinogenesis. The rejection of fast-growing transplantable tumors requires high-avidity cytotoxic T lymphocytes that DNA vaccination fails to elicit in transgenic BALB-neuT mice. In addition, these data show that specific vaccination manages the aggressive progression of HER-2/*neu* mammary carcinogenesis more effectively than the natural reactivity elicited by systemic IL-12 [16]. The best IL-12 regimen markedly delays, but rarely inhibits this carcinogenesis [17]. The mechanisms involved are also different. IL-12 induces the release of a series of downstream mediators that trigger natural cell immunity and impair the vascular proliferation associated with carcinogenesis. By contrast, ECD-TM DNA vaccination results in inhibited progression unaccompanied by areas of ischemic-hemorrhagic necrosis or signs of vascular damage.

The progressive delay in the beginning of DNA vaccination goes along with a progressive decrease in its protective ability.

How can the efficacy of these vaccinations be prolonged?

The observation time for the above vaccination experiment was limited to 33 weeks. What happens later on? When we repeated the experiment and extended the observation period to 1 year, the final protection was much less impressive (Fig. 6). After 1 year, only a minority of the animals is still protected. Vaccination results in a significant delay but its final effect is not very impressive.

Prolongation of immune memory is thus a more worthwhile goal than enhancement of the immune response. How can we preserve an active memory throughout the natural progression of HER-2/*neu* tumors? We are currently evaluating the ways in which this can be done. The most attractive approach so far has been the injection of soluble LAG-3 simultaneously with

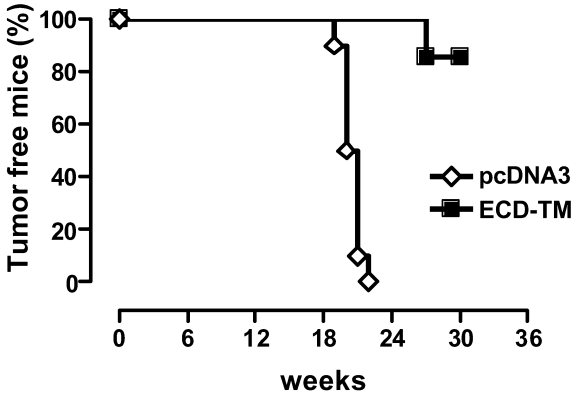

Fig. 5 Inhibition of mammary carcinogenesis in BALB-neuT mice at 33 weeks of age. Mice are vaccinated intramuscularly at the 4th and the 7th week with 100 μg of pcDNA3 or ECD-TM plasmid. Percentages of tumor-free mice are calculated as the cumulative number of incident tumors / total number of mice

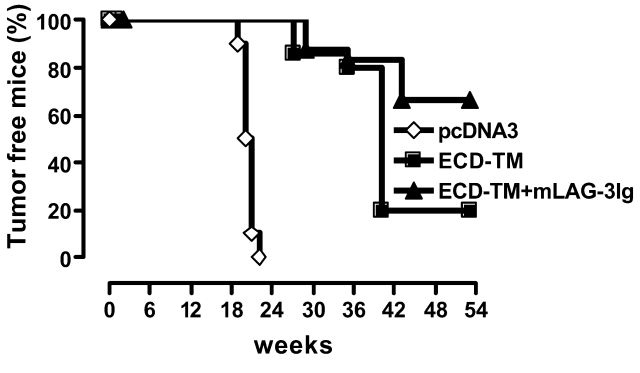

Fig. 6 Longer protection by DNA vaccination and mLAG-3Ig. BALB-neuT mice were immunized with 100-μg ECD-TM plasmid alone or in association with 20 μg of mLAG-3Ig. At 52 weeks of age, 20% of mice vaccinated with ECD-TM and about 80% of those with ECD-TM + mLAG-3Ig are tumor free

DNA vaccination. LAG-3 (lymphocyte activation gene 3 / CD223) is a type 1 transmembrane protein associated with TCRs. It is independent of, but very similar to, CD4 [47] and binds class II major histocompatibility antigens. Association of plasmid vaccination with administration of soluble mouse LAG-3 generated by fusing the extracellular domain of mLAG-3 to a murine IgG2a Fc portion (mLAG-3Ig) elicited stronger and more sustained protection that kept almost 70% of 1-year-old mice tumor free (Fig. 6) [11]. Moreover, this combined vaccination performed when multiple in situ carcinomas were already evident, extended disease-free survival, and reduced carcinoma multiplicity. Inhibition of carcinogenesis was associated with markedly reduced epithelial cell proliferation and rat p185neu expression, while the few remaining hyperplastic foci were heavily infiltrated by reactive leukocytes. Stronger and enduring rat p185neu–specific cytotoxicity, sustained release of IFN-γ and IL-4, and marked expansion of both $CD8^+CD11b^+CD28^+$ effector and $CD8^+CD11b^+CD28^-$ memory effector T-cell populations were induced in immunized mice. This combined vaccination also elicited a quicker and higher antibody response to rat p185neu, as well as an early antibody isotype switch. These data suggest that the appropriate costimulation provided by LAG-3Ig enables DNA vaccination to establish effective protection, probably by enhancing cross-presentation of the DNA coded antigen.

Similar results are now being obtained with the association of ECD-TM vaccination and treatment with systemic recombinant IL-12 (Spadaro et al., manuscript in preparation).

How can the efficacy of these vaccinations be increased?

Can we also use DNA vaccination against a more advanced preneoplastic lesion? When the beginning of DNA vaccination was postponed until BALB/c mice already displayed multifocal lesions in all ten mammary glands and displayed a situation more or less equivalent to that of a multifocal carcinoma in situ, DNA vaccination was only marginally effective. Tumor onset was delayed. Some mice were protected, but this was not significant protection. All ten glands displayed a palpable tumor in the control animals, whereas the percentage was lower in the vaccinated mice.

Improvement of DNA vaccination is now being attempted by using different approaches, including the association of vaccination with LAG-3Ig, recombinant IL-12, BAT monoclonal antibodies [28], or boosting DNA-vaccinated mice with rat p185neu–positive allogeneic cells engineered to secrete IFN-γ. While all these studies are currently under way, the best method to date for improving DNA vaccination has been to associate it with electrical pulses, performing what is known as electroporation (Fig. 7). In this case, 25 μg of p185 plasmids is first injected bilaterally into the tibial muscle of anesthetized BALB-neuT mice. Transcutaneous electric pulses are then applied by two stainless steel plate electrodes placed 3 mm apart on the shaved skin covered by a conductive gel. Square-wave electric pulses are generated by a T820 electroporator (BTX, San Diego, CA). Two electric pulses of 25 ms with an electric-field strength of 375 V/cm are administered. When this electroporation was performed on BALB-neuT mice already displaying multifocal lesions in all ten mammary glands, 43% of them were still completely tumor free at week 52 (Quaglino et al., manuscript in preparation). The aggressive and inexorable nature of HER-2/*neu* carcinogenesis in BALB-neuT mice makes this persistent inhibition of the progression of multiple and large subclinical neoplastic lesions a very significant finding.

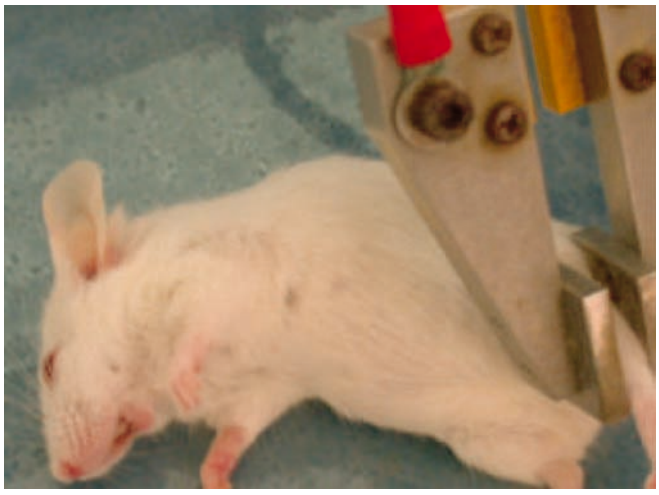

Fig. 7 The T820 electroporator was used to improve DNA uptake. Two minutes after DNA injection, we covered the legs of the anesthetized mouse with a conductive gel, and then applied electrodes at the end of the caliper to the leg muscle. We used two electric pulses of 25 ms each and at an intensity of 375 V/cm

Immune reactivity associated with DNA-vaccination–induced inhibition of carcinogenesis

Pathological observations

Protection from carcinogenesis is associated with an initial inhibition of the progression of mammary lesions followed by a slow but progressive clearance. In vaccinated mice, both the multiple side buds and the large agglomerates of rat p185neu-positive cells present at the time of vaccination were still unchanged at week 15, while they were markedly reduced by 21 weeks. The cells of these residual neoplastic lesions no longer expressed membrane rat p185neu. It was only expressed in the cytoplasm, but even there its expression was fainter than in untreated BALB-neuT mice [11, 39]. At week 52, side buds as well as rat p185neu cells, had vanished in the mammary glands of the DNA electroporated mice. This clearance was associated with a simplified fractal pattern

of ductal ramification, while little or no reactive cell infiltrate was evident. Only in a few mammary glands, residual and constrained foci of hyperplasia associated with a distinct reactive cell infiltrate were still present close to the nipple region. By contrast, large neoplastic agglomerates persisted in the mammary glands of intramuscular vaccinated mice.

The role of antibodies

Antibodies are usually regarded as being of marginal or even of no importance in tumor inhibition. We have thus been increasingly intrigued by the constant production of anti-rat p185neu antibodies when HER-2/*neu* carcinogenesis is inhibited in BALB-neuT mice vaccinated with DNA [11, 39, 40]. When incubated with TUBO cells, sera from immune mice stripped rat p185neu from their membrane and internalized it in the cytoplasm (Fig. 8). A similar impressive down-modulation of rat p185neu membrane expression was observed in the hyperplastic lesions present in BALB-neuT mice at the time of ECD-TM immunizations. The reduced membrane expression of rat p185neu that follows immunization was accompanied by diminished nuclear positivity of PCNA, a marker associated with cell proliferation [16]. These findings suggest that anti-p185neu antibodies react against and down-modulate the expression of a growth factor receptor causally implicated in carcinogenesis. Rat p185neu down-modulation appears to slow preneoplastic cell proliferation and tumor development. This antibody inhibition of neoplastic progression is different from direct immunological destruction of malignant cells. However, in immunized mice, leukocytes at the tumor growth site may also play an important regulatory role. Anti-rat p185neu antibodies may activate polymorphonuclear granulocytes and other cells to engage in antibody-dependent cell-mediated cytotoxicity [7] and complement-dependent cytotoxicity (IgG2a and IgM), and inhibit the growth of rat p185neu–positive tumors in vivo.

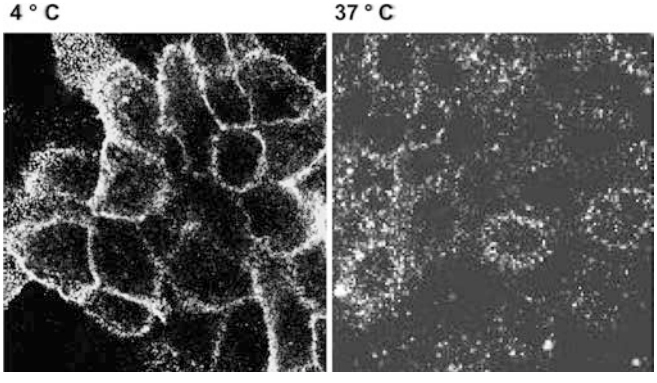

Fig. 8 Sera of BALB-neuT–vaccinated mice have specific anti-p185neu antibodies able to down-regulate rat p185neu from the membrane of TUBO cells. When TUBO cells are incubated with Abs specific for p185neu at 37°C the receptor is internalized while at 4°C the down-regulation is blocked, as indicated by confocal microscopy studies. Their activity appears to be similar to that of passively administered anti-rat p185neu monoclonal antibodies

This important role of antibodies in the blockade of HER-2/*neu* carcinogenesis is not surprising, since it is well known that monoclonal antibodies to p185 induce a functional block of p185 receptor function, down-regulate its expression on the cell membrane, impede its ability to form homodimers or heterodimers that spontaneously transduce proliferative signals to the cells, and block its ability to bind ligands (reviewed in [49]). These antibodies also significantly suppress the growth of transplantable p185neu-positive tumors, and delay tumor growth in patients with HER-2/*neu*-positive tumors. Herceptin or trastuzumab is one of the anti-ErbB-2 monoclonal antibodies that has clinical efficacy in metastatic breast cancer. However, despite Herceptin's success, it is still not perfectly clear how monoclonal antibodies inhibit tumor growth in vivo, and diverse mechanisms of action have been proposed. One of the first mechanisms of action attributed to inhibitory monoclonal antibodies is the removal of p185neu from the cell's surface. This normally coincides with antibody internalization. Clearly, down-regulation of p185neu could induce growth inhibitory effects on its own by decreasing the signaling efficiency of the entire signaling network [49]. The down-regulation could result in the disruption of p185neu homodimer or heterodimer formation and consequently decreased receptor phosphorylation and catalytic activity. Herceptin is able to prevent the cleavage of p185neu ECD (which leads to receptor constitutive activation) and to recruit host immune effector cells [4]. Less understood are the downstream events that occur following the interaction between p185neu and anti-p185neu antibodies. Antibodies can interfere with specific programs of gene expression, inducing a transient increase in c-*fos* mRNA expression [41] or the recovery of a functional TBP-1, a tumor suppressor–like gene [37] and can alter normal cell cycle progression [31]. Inhibition of tumor growth could also result from the ability of anti-p185neu antibodies to induce tumor cell apoptosis [32].

All these studies have been performed using monoclonal antibodies that recognize a single epitope. However, it has been shown that the epitope recognized on p185neu directs the biological activity of the antibody [50]. For example, Herceptin is not able to inhibit signaling by ligand-induced ErbB-2–containing heterodimers, a clearly important mechanism of receptor activation [4]. On the contrary, the anti-ErbB-2 monoclonal antibody 2C4 that binds to a different epitope in the p185neu ECD than Herceptin, blocks the association of ErbB-2 with other members of the EGFR family [1]. In DNA-vaccinated BALB-neuT mice there is a polyclonal production of anti-rat p185neu antibodies, and various epitopes of the rat p185neu are recognized (Lanzardo et al., in preparation) leading to the simultaneous activation of distinct reaction mechanisms. Our ongoing experiments aim:

a. to clarify the downstream events occurring after p185neu internalization;
b. to identify the dominant epitopes of rat p185neu recognized by the sera of DNA-vaccinated BALB-neuT mice;
c. to assess the importance of the antibody-mediated mechanisms in halting mammary carcinogenesis in BALB-neuT mice.

Due to the early and diffuse overexpression of rat p185neu, BALB-neuT mice are probably unable to produce high-affinity anti-rat p185neu antibodies. Nevertheless, the slow progression of carcinogenesis allows poor-avidity antibodies to play a significant inhibitory role. While antibodies to rat p185neu cross-react with mouse p185neu, the absence of autoimmune reactions against tissues expressing low numbers of mouse p185neu molecules is probably the reward for not being able to produce high-affinity responses.

Conclusions

These results suggest that the progression of neoplastic lesions due to a specific gene alteration can be prevented immunologically. This possibility opens up prospects for novel strategies in cancer prevention. Cytokine boosting of natural immunity is a means of hampering carcinogenesis in the absence of identification of the target antigen. During HER-2/*neu* carcinogenesis, specific recognition of p185neu epitopes by noncytotoxic T cells and antibodies, and ADCC may lead to even more effective protection. Immune mechanisms elicited by vaccination can definitively save these mice from the multiple mammary carcinomas to which they are genetically predisposed.

These results suggest that immunomodulation could be envisaged as an effective new prospect in the prevention of carcinogenesis in humans. However, despite the similarity of mammary carcinogenesis in BALB-neuT mice and women, the data from BALB-neuT transgenic mice cannot be directly translated to humans because the mechanisms of tolerance to self-p185neu in women could be different from those of rat p185neu in transgenic mice, and the escape mechanisms of human preneoplastic lesions may be more difficult to overcome.

It should be emphasized that immune control of preneoplastic lesions appears much more effective than inhibition of small established tumors. Slow-growing preneoplastic lesions can thus be seen as susceptible to slow and low-affinity immunoreactions elicited in tolerant mice, whereas these same reactions are not enough to inhibit the growth of fast-growing transplantable tumors.

On the other hand, p185neu can be considered as the prototype of a class of TAAs of major importance because it is expressed on preneoplastic lesions and its expression is instrumental for tumor progression [33]. Not only is it a TAA, but also the product of oncogenes causally involved in neoplastic progression. Their early expression along with the limited proliferation of preneoplastic lesions makes them a relatively stable tag. The chance of an immune reaction selecting clones in a preneoplastic lesion whose progression is independent of HER-2/*neu* appears unlikely, because the identity between oncogene product and TAA ensures that TAA-loss variants will not grow until many additional mutations have made a clone independent of the HER-2/*neu* transduction pathway. While this may happen in large tumors, in preneoplastic lesions p185neu-loss clones can only elude the immune response by losing their ability to give rise to progressively growing tumors.

In conclusion, can these experimental data endorse preventive vaccination? Can we imagine a form of vaccination that inhibits the progression of preneoplastic lesions or prevents tumors in patients at risk?

The first and simplest application of preventive vaccination is in the prevention of tumor recurrences. However, this is a special form of prevention, because many of the theoretical reasons that make preventive vaccination appealing are not fulfilled: the immune system has already seen the tumor and the tumor antigen presented in nonantigenic surroundings. Nevertheless, this will probably be the first situation subjected to clinical evaluation. A second situation that is more closely related to "true" prevention is the employment of vaccination to impair the progression of preneoplastic lesions. Could vaccination be used in this case? The limit of this approach is that one must be absolutely sure that vaccination will not elicit side effects worse than the protection provided. There are also a few situations in which genetic screenings can detect a population at a specific risk of cancer and where specific vaccination could be contemplated. We also have to consider that there are now many ways of determining a person's risk of cancer. Predictive oncology can forecast the kind of tumors most likely to appear in certain populations. Finally, a thought could also be given to the widespread vaccination of completely healthy persons, as everyone has a generic risk of developing cancer. In this case, we are leaving the world of data to enter the land of scientific dreams.

References

1. Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, Lofgren JA, Tindell C, Evans DP, Maiese K, Scher HI, Sliwkowski MX (2002) Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell 2:127
2. Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? Lancet 257:539
3. Bargmann CI, Weinberg RA (1988) Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. Proc Natl Acad Sci U S A. 85:5394
4. Baselga J (2001) Herceptin alone or in combination with chemotherapy in the treatment of HER2-positive metastatic breast cancer: pivotal trials. Oncology 61:14

5. Bishop JS, Thull NM, Matar M, Quezada A, Munger WE, Batten TL, Muller S, and Pericle F (2000) Anti-tumoral effect of a non-viral interleukin-2 gene therapy is enhanced by combination with 5-fluorouracil. Cancer Gene Ther 7:1165
6. Blezinger P, Freimark BD, Matar M, Wilson E, Singhal A, Min W, Nordstrom JL, Pericle F (1999) Intratracheal administration of interleukin 12 plasmid-cationic lipid complexes inhibits murine lung metastases. Hum Gene Ther 10:723
7. Bocchia M, Bronte V, Colombo MP, De Vincentiis A, Di Nicola M, Forni G, Lanata L, Lemoli RM, Massaia M, Rondelli D, Zanon P, Tura S (2000) Antitumor vaccination: where we stand. Haematologica 85:1172
8. Boggio K, Nicoletti G, Di Carlo E, Cavallo F, Landuzzi L, Melani C, Giovarelli M, Rossi I, Nanni P, De Giovanni C, Bouchard P, Wolf S, Modesti A, Musiani P, Lollini PL, Colombo MP, Forni G (1998) Interleukin-12 mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/*neu* transgenic mice. J Exp Med 188:589
9. Boggio K., Di Carlo E., Rovero S, Cavallo F, Quaglino E, Lollini P-L, Nanni P, Nicoletti G, Wolf S, Musiani P, Forni G (2000) Ability of systemic IL-12 to hamper progressive stages of mammary carcinogenesis in Her2/neu trangenic mice. Cancer Res 60:359
10. Boon T (1983) Antigenic tumor cell variants obtained with mutagens. Adv Cancer Res 39:121
11. Cappello P, Triebel F, Iezzi M, Caorsi C, Quaglino E, Nanni P, Amici A, Di Carlo E, Musiani P, Giovarelli M, Forni G (2003) LAG-3 enables DNA vaccination to persistently prevent mammari carcinogenesis in HER-2/neu transgenic BALB/c mice. Cancer Res 63:2518
12. Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM, Ward JM, Green JE (2000) The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene 19:968
13. Cavallo F, Di Pierro F, Giovarelli M, Gulino A, Vacca A, Stoppacciaro A, Forni M, Modesti A, Forni G (1993) Protective and curative potential of vaccination with interleukin-2-gene-transfected cells from a spontaneous mouse mammary adenocarcinomas. Cancer Res 53:5067
14. Cavallo F, Signorelli P, Giovarelli M, Musiani P, Modesti A, Brunda MJ, Colombo MP, Forni G (1997) Antitumor efficacy of adenocarcinoma cells engeneered to produce interleukin 12 (IL-12) or other cytokines compared with exogeneous IL-12. J Natl Cancer Inst 89:1049
15. Cavallo F, Di Carlo E, Butera M, Verrua R, Colombo MP, Musiani P, Forni G (1999) Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12. Cancer Res 59:414
16. Cavallo F, Quaglino E, Cifaldi L, Di Carlo E, Andrè A, Bernabei P, Musiani P, Forni G and Calogero RA (2001) IL12-activated lymphocytes influence tumor genetic programs. Cancer Res 61:3518
17. Cifaldi L, Quaglino E, Di Carlo E, Musiani P, Spadaro M, Lollini P-L, Wolf S, Boggio K, Forni G, Cavallo F (2001) A light, nontoxic IL-12 protocol inhibits Her2/neu mammary carcinogenesis in BALB/c transgenic mice with established hyperplasia. Cancer Res 61:2809
18. Coughlin CM, Salhany KE, Gee MS, LaTemple DC, Kotenko S, Ma X, Gri G, Wysocka M, Kim JE, Liu L, Liao F, Farber JM, Pestka S, Trinchieri G, Lee WM (1998) Tumor cell rresponse to IFN-gamma affect tumorigenicity and response to IL-12 therapy and angiogenesis. Immunity 9:25
19. Coussens LM, Werb Z (2001) Inflammatory cells and cancer: think different! J Exp Med 193:F23
20. Curcio C, Di Carlo E, Clynes R, Smyth MJ, Boggio K, Quaglino E, Spadaro M, Colombo MP, Amici A, Lollini PL, Musiani P, Forni G (2003) Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. J Clin Invest 111:1161
21. De Stefani A, Forni G, Ragona R, Cavallo F, Bussi M, Usai A, Badellino F, Cortesina G (2002) Improved survival with perilymphatic interleukin 2 in patients with resectable squamous cell carcinoma of the oral cavity and oropharynx. Cancer 95:90
22. Di Carlo E, Diodoro MG, Boggio K, Modesti A, Modesti M, Nanni P, Forni G, Musiani P (1999) Analysis of mammary carcinoma onset and progression in HER-2/neu oncogene transgenic mice reveals a lobular origin. Lab Invest 79:1261
23. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3:991
24. Forni G, Lollini P-L, Musiani P, Colombo MP (2000) Immunoprevention of cancer: is the time ripe? Cancer Res 60:2571
25. Garcia-Lora A, Algarra I, Collado A, Garrido F (2003) Tumour immunology, vaccination and escape strategies. Eur J Immunogenet 30:177
26. Grimm EA (1986) Human lymphokine-activated killer cells (LAK cells) as a potential immunotherapeutic modality. Biochim Biophys Acta 865:267
27. Gullick WJ, love SB,Wright C, Barnes DM, Gusterson B, Harris AL (1991) C-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer 63:434
28. Hardy B, Yampolski I, Kovjazin R, Galli M, Novogrodsky A (1994) A monoclonal antibody against a human B lymphoblastoid cell line induces tumor regression in mice. Cancer Res 54:5793
29. Klein G, Sjogren HO, Klein E (1960) Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. Cancer Res 20:1561
30. Koido S, Kashiwaba M, Chen D, Gendler S, Kufe D, Gong J (2000) Induction of antitumor immunity by vaccination of dendritic cells transfected with MUC1 RNA. J Immunol 165:5713
31. Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hjnes NE (2000) ErbB2 potentiates breast tumor proliferation through modulation of p27 (Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. Mol Cell Biol 20:3210
32. Lewis Phillips GD, McMurtrey AE, Schroeder K, Fendly BM (1998) Diverse activities of anti-HER2 monoclonal antibodies: from growth inhibition to induction of apoptosis. Proc Am Assoc Cancer Res 39:143
33. Lollini P-L, De Giovanni C, Nicoletti G, Di Carlo E, Musiani P, Nanni P, Forni G (2002) Immunoprevention of colorectal cancer: a future possibility? Gastroenterol Clin North Am 31:1001–14
34. Muller WJ, Sinn E, Wallance R, Pattengale PK, Leder P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 54:105
35. Nanni P, Nicoletti G, De Giovanni C, Landuzzi L, Di Carlo E, Cavallo F, Pupa SM, Rossi I, Colombo MP, Ricci C, Astolfi A, Musiani P, Forni G, Lollini P-L (2001) Combined allogeneic tumor cell vaccination and systemic IL-12 prevents mammary carcinogenesis in HER-2/neu transgenic mice. J Exp Med 194:1195–1205
36. Noguchi Y, Jungbluth A, Richards EC, Old LJ (1996) Effect of interleukin 12 on tumor induction by 3-methylcholanthrene. Proc Natl Acad Sci U S A 93:11798–11801
37. Park BW, O'Rourke DM, Wang Q, Davis JG, Post A, Qian X, Greene MI (1999) Induction of the Tat-binding protein 1 gene accompanies the disabling of oncogenic erbB recepotr tyrosine kinases. Proc Natl Acad Sci U S A 96:6434
38. Qin Z, Richter G, Schuler T, Ibe S, Cao X, Blankenstein T (1998) B cells inhibit induction of T cell-dependent tumor immunity. Nat Med 4:627
39. Rovero S, Amici A, Carlo ED, Bei R, Nanni P, Quaglino E, Porcedda P, Boggio K, Smorlesi A, Lollini PL, Landuzzi L, Colombo MP, Giovarelli M, Musiani P, Forni G (2000) DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. J Immunol 165:5133

40. Rovero S, Boggio K, Di Carlo E, Amici A, Quaglino E, Porcedda P, Musiani P, Forni G (2001) Insertion of the DNA for the 163–171 peptide of IL1β enables a DNA vaccine encoding p185 (neu) to inhibit mammary carcinogenesis in Her-2/neu transgenic BALB/c mice. Gene Ther 8:447
41. Scott GK, Dodson JM, Montgomery PA, Johnson RM, Sarup JC, Wong WL, Ullrich A, Shepard HM, Benz CC (1991) p185her2 signal transduction in breast cancer cells. J Biol Chem 266:14300
42. Shau H, Kim A, Golub H (1992) Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. J Leukocyte Biol 51:343
43. Shih C, Padhy LC, Murray M, Weinberg RA (1981) Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature 290:261
44. Smith RT, Landy M (eds) (1970) Immune surveillance. Academic Press, New York
45. Smyth MJ, Trapani JA (2001) Lymphocyte-mediated immunosurveillance of epithelial cancers? Trends Immunol 22:409
46. Strasly M, Cavallo F, Geuna M, Mitola S, Colombo MP, Forni G, Bussolino F (2001) IL-12 inhibition of endothelial cell functions and angiogenesis depends on lymphocyte-endothelial cell cross-talk. J Immunol 166:3890
47. Triebel F, Jitsukawa S, baixeras E, Roman-Roman S, Genevee C, Viegas-Pequinot E, Hercend T (1990) LAG-3, a novel lymphocyte activation gene related to CD4. J Exp Med 171:1393
48. Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev 3:133
49. Yip YL, Ward RL (2002) Anti-ErbB-2 monoclonal antibodies and ErbB-2-directed vaccines. Cancer Immunol Immunother 50:569
50. Yip YL, Smith G, Koch J, Dubel S, Ward RL (2001) Identification of epitope regions recognized by tumor inhibitory and stimulatory anti-erbb-2 monoclonal antibodies: implications for vaccine design. J Immunol 166:5271