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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our program was to evaluate the ability of non specific and specific immunity to hamper the carcinogenesis. The ultimate goal was to determine whether this type of approach can be rationally applied in humans at risk. The model used consisted in virgin female mice transgenic for the activated (BALB-neuT) or amplified (FVB-neuN) rat Her-2/neu oncogene. BALB-neuT mice progress rapidly to multiple mammary carcinomas, while in FVB-neuN carcinogenesis is slow. A timely administration of low doses of IL-12 elicits in a complex immunological reaction that markedly delay the progression of carcinogenesis and alter the angiogeneic capacity of carcinomas. This prevention by delaying takes place in the absence of any antigenic definition. A much more effective and persistent prevention can be induced by specific immunization against the Her-2/neu membrane product ($p185^{neu}$). A strong protection was afforded by vaccination with DNA plasmids coding for $p185^{neu}$, while no protection was elicited by vaccination with distinct $p185^{neu}$ peptides. $p185^{neu}$ positive allogeneic cells are effective vaccines and their immunogenicity is further increased when these cells are engineered to release IFN-γ. All mice vaccinated with allogenic $p185^{neu}$ cells and receiving systemic IL-12 are tumor free at one year of age. The combination of nonspecific and specific immunostimulation appears to be an effective way to protect the host form an ongoing carcinogenic process.

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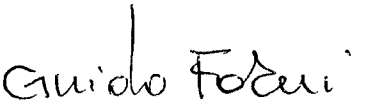
Foreword

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

- In conducting research using animal, the investigators adhered to the "Guide for Care and Use of Laboratory Animals" , prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).



Signature of Guido Forni

Principal Investigator

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5 INTRODUCTION

Breast cancer is the most frequent malignancy of women worldwide. Therefore, in many communities genetic screening and tumor prevention programs are leading to the identification of an increasing number of woman at risk or bearing prenenoplastic lesions. Unfortunately, once that diagnosis is made, therapeutic options rest largely on successive evaluations of the disease's progress and radical mastectomy often associated with ovariectomy. In the absence of firm therapeutic options, frequent screenings to assess the disease progression activate anxiety, while no less devastating are the maladaptive thoughts related to radically mutilating prophylactic surgery. The importance of preventive interventions providing effective and soft options is evident. Rationally many considerations suggest that inhibition of carcinogenesis is an appropriate immunological goal (Table I).

Table I. Cancer immunoprevention: *Guiding principles:*

- Response to immunization inversely correlates with total body burden of cancer
- Carcinogenesis is a slow process
- This slowness leaves open the time window required for the induction of an immune response Healthy individuals are more likely to mount an effective immune response
- Immunosuppression correlates with tumor stage
- Immune mechanisms are likely to have greater impact on subclinical disease (1)

From the suggestions provided by Dr. Jaye L. Viner, NCI, Division of Cancer Prevention, MD. USA

NONSPECIFIC IMMUNITY IN CANCER PREVENTION

The selection of not-yet patients and healthy individuals eligible for immunoprevention depends on the kind of treatment envisaged. Enhancement of nonspecific immunity and specific antitumor vaccination are two possible approaches. The advantage of a nonspecific antitumor response is that it can be directly applied to a broad range of individuals, irrespective of the type of TAA their foreseeable tumors may eventually express. However, it is unfeasible to imagine healthy persons being

nonspecifically treated for long periods. The results of the mouse experiments indicate that nonspecific stimulation should thus be restricted to non-yet patients with a genetic risk of cancer (2), individuals exposed to high carcinogen doses (3), patients with a preneoplastic lesion and those that probably have minimal residual disease after a successful conventional treatment. Many not-yet patients with a high risk of cancer are, in fact, being recruited in ongoing programs to screen for preneoplastic lesions or gene mutations that predispose to cancer. Women at risk for breast cancer or with preneoplastic lesions form a category for which nonspecific immunoprevention could be considered as a practical option.

SPECIFIC IMMUNITY IN CANCER PREVENTION.

Specific vaccination of persons at risk and healthy individuals constitutes a very different scenario. Characterization of specific gene alterations or detection of preneoplastic lesions may indicate which organ and tissue are at risk. In a few cases, more precise information may show which oncogene product will probably be overexpressed or expressed in an altered form and allow vaccination against a single, specific TAA. Molecular characterization of altered gene products predictably destined to become TAA will be the first step towards the engineering of selective vaccines. Otherwise, the patient should be vaccinated against the TAA most commonly expressed by the tumors foreseeable in a given organ.

Many new antitumor vaccines that induce an effective resistance to subsequent tumor challenge and inhibit minimal residual disease are already available (4). The question whether specific immunization can be successful once a cell population has been subjected to the initial carcinogenic hit has rarely been examined experimentally. It can, however, be plausibly suggested that cytokines and more conventional adjuvants could induce an effective immune response against ignored or fully tolerated antigens

6. BODY

THE EXPERIMENTAL MODEL

There are distinct models that allow to approach the features of mammary carcinogenesis in women with different approximation. Quite often transplantable tumors are used even if the information provided by these models is questionable.

Table II. Transplantabe tumors

Most experimental data (and beliefs) are from transplantable tumors. These:

- Are easy to standardize & handle
- Provide reproducibility of data
- Make well-defined experimental systems

- Give rise to highly artifactual models
- Display a quick growth kinetics
- Growth in "healthy" recipients
- Establish unnatural tumor-host relationship

The system we have build up to study this problem consist in inbred BALB/c mice transgenic for the activating rat Her-2/neu oncogene (BALB-neuT) (Fig.1). We established these mice starting from a non-inbred Her-2/neu transgenic mouse after about three years of backcrossig with BALB/c mice. The BALB-neuT females are interesting since they present a spontaneous progression from normal mammary gland to atypical hyperplasia, carcinoma in situ and invasive and metastasizing carcinoma that is consistent and mimics what is observed in women (5). These BALB-neuT mice formed the basic experimental model used in the studies performed during this research program.

In a few cases the results obtained in BALB-neuT mice were compared with those obtained on FVB mice transgenic for the Her-2/neu protoncogene, that display a much less aggressive mammary carcinogenesis (5).

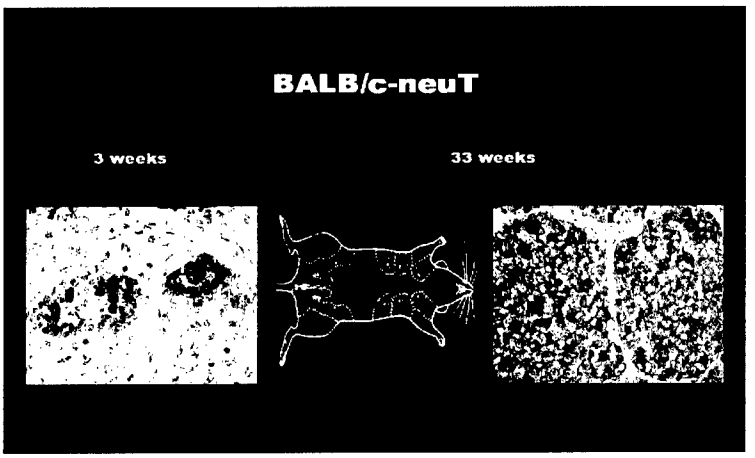

Fig. 1 *Key features of our BALB-neuT female mice.* ***A. p185^{neu} is a tolerated antigen****: The rat Her-2/neu oncogene coding for the p185^{ne} is markedly overexpressed in the rudimental mammary gland of 3-week-old mice. In red, positivity to anti rat p185^{neu} (left panel).* ***B. Very aggressive carcinogenesis:*** *At 33 weeks all ten mammary glands display palpable tumors (central panel).* ***C. Expression of the target antigen.*** *These tumors are lobular carcinomas markedly stained by anti rat p185^{neu} (right panel).*

TASK 1. PREVENTION OF MAMMARY CARCINOMA WITH IL-12.

Goals

The specific aim is to prevent mammary carcinoma in BALB-neuT and FVB-neuN female mice by repeated systemic administrations of IL-12 and to understand the mechanisms involved. These two lines of transgenic mice express the rat HER-2/neu oncogene under the transcriptional control of mouse mammary tumor virus (6). Female BALB-neuT mice show no morphological abnormalities of the mammary gland until 3 wk of age. They then progress through atypical hyperplasia to in situ lobular carcinoma and at 33 wk of age all ten mammary glands display invasive carcinomas (Fig.1). In adult FVB-neuN mice (H-2^{q}) carrying the HER-2/neu protooncogene, neoplastic progression is less impetuous, as shown by a longer latency (38-49 weeks) and a lower tumor multiplicity (mean of 2.6 tumors/mouse).

In vivo effects

Treatment with IL-12 (five daily i.p. injections, 1 week on, 3 weeks off; the first course with 50 ng IL-12/day, the following with 100 ng IL-12/day) begun at the 2nd week of age in BALB-neuT mice and at the 21st week in FVB-neuN mice markedly delayed tumor onset and reduced tumor multiplicity. In both mouse lines, tumor inhibition was associated with deficient peri- and intra-tumoral angiogenesis, infiltration of reactive cells, production of pro-inflammatory cytokines, and inducible NO synthetase activation (5).

IL-12 efficacy in progressive stages of carcinogenesis

We therefore set out to define the progression stage in which IL-12 induced reaction mechanisms are most effective. Should IL-12 administration be proposed as a preventive measure in not-yet patients only, or can it also be of benefit once overt preneoplastic lesions are diagnosed? This is a significant question since genetic screening programs are singling out healthy not-yet patients and early diagnosis programs are detecting pre-neoplastic lesions (7). Technical and specific features related to this task of the program are reported in detail in Appendices. Groups of BALB-neuT and FVB-neuN mice received IL-12 at progressive times during carcinogenesis. IL-12 (Genetics Institute, Cambridge, MA) in Hank's balanced salt solution supplemented with 0.01% mouse serum albumin (MSA, Sigma, St. Louis, MO) was administered intraperitoneally. Mice received seven 5-day courses of MSA only (MSA controls) or MSA plus IL-12. Other groups of mice remained untreated. To evaluate the ability of IL-12 to inhibit this progression, mice received seven 5-day courses of IL-12 at different times. As BALB-neuT mice already display hyperplasia of small lobular ducts and lobules at 3 weeks of age, "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 of the number of mammary glands with a palpable tumor at 33 weeks when the experiment ended were observed as compared to MSA controls. To assess whether IL-12 is also effective during later phases, other 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 none the less reduced the number of tumors at week 33 by 22%. The "Early" treatment began at the 2nd week and continued until week 14. The delay of first tumor onset and the reduction of the number of tumor are significantly higher than in "Chronic" treatment. When the "Early" treatment was further split into shorter four-week administration schedules, much less protection was observed (8).

Dose of IL-12

In addition to timing, the IL-12 dose 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 previous findings to a clinical setting suggested that IL-12 treatment may 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 also explored whether a much lower dose of IL-12 started when adult mice already present full-blown atypical mammary hyperplasia is as efficacious as much earlier and heavier treatments (5). The aggressive mammary carcinogenesis that takes place in all the mammary glands of BALB-neuT female mice was significantly hampered in those receiving sixteen i.p. administrations of 100 ng IL-12 divided into four courses of weekly injections for four weeks followed by a three 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 the effective tumor inhibition, in another set of experiments BALB-neuT mice received only the first

two or three courses of IL-12. 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 previously observed with 5 injections per week (5). Since the ability of IL-12 to elicit high levels of IFN-γ correlates with the clinical response, serum IFN-γ levels after a single and multiple IL-12 injections were compared. Within 6 to 120 hours after a single IL-12 injection the IFN-γ titers were much higher than for the same period after the fifth IL-12 injection. Moreover, while 6 hours after one or two IL-12 injections high titers of IFN-γ were found, these dropped progressively by further increasing the number of IL-12 injections. However, when mice that received one IL-12 injection were boosted 7 and 14 days later, the levels of IFN-γ decreased. In conclusion, an 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 a 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 (8). 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 (9).

The use of IL-12 in humans is complicated by schedule- and dose-dependent toxicity. Weekly administration of 500 ng/kg is well tolerated by melanoma patients. It is effective clinically and transiently boosts tumor-specific T lymphocytes. Unfortunately, even with the present light schedule, the single dose effective in mice is still eight-ten times higher than the 500-700 ng/kg tolerated by humans and lower single doses are almost ineffective. For other cytokines (e.g. IL-2), increased experience and refinements in patient selection and administration schedules have greatly increased the safety of antitumor regimens. In any event, cancer prevention is another matter and comparison with the doses used in cancer therapy may be inappropriate.

IL-12 inhibition of angiogenic switch

The efficacy of IL-12 in BALB-neuT mice suggests that evolution of the tumor:host angiogenic relationship, rather than intrinsic proliferative properties of transformed mammary cells is the point of no return for its activity. At least part of this antitumor activity appears to depend on its ability to inhibit the angiogenesis associated with mammary hyperplasia. Immunohistochemical staining with anti-CD31 monoclonal antibody shows that rich microvascularisation inside preneoplastic lesions corresponds to their progression towards carcinoma, as shown in other tumor systems. This progression phase appears to be particularly appropriate for an angiostatic intervention.

The importance of the time of IL-12 administration was further assessed with FVB-neuN mice, in whom an overexpressed Her-2/*neu* protooncogene induces mammary carcinomas after a markedly longer latency. The "6-week-old" treatment consists in a lifetime administration of IL-12 and is conceptually similar to the "Chronic" treatment of BALB-neuT mice. While on the "22-week-old" treatment the first course was markedly delayed, it still started before an evident spreading of preneoplastic lesions. Both treatment schedules delay the onset of carcinomas and their multiplication. The period between the 22nd and the 28th week appears to be of critical importance, as the "28-week-old" protocol confers a negligible protection only. During these six weeks, in fact, normal mammary glands progress towards atypical hyperplasia and then to carcinoma in situ and invasive carcinoma.

The equivalent results from BALB-neuT and FVB-neuN mice suggest that IL-12 effectively inhibits mammary carcinogenesis when its administration accompanies the angiogenic switch. Its anti-angiogenic effect appears to rest on the increased serum levels of IFN-γ and TNF-α released by activated T lymphocytes and NK cells, whose anti-angiogenic and angiotoxic activity is stronger on the fragile capillary sprouts that accompany the shift from the preneoplastic to the neoplastic condition. Downstream mediators elicited by IL-12 may also act on neoplastic cells in which they down regulate the production of pro-angiogenic molecules and up regulate the release of anti-angiogenic factors, such as IP-10 and MIG. Following the transition from hyperplasia to in situ and invasive carcinoma, capillary sprouting is restrained. The poor efficacy of late treatment

may depend on the lower sensitivity to IL-12-induced angiostasis of the mature and differentiated blood vessels of the advanced neoplastic lesions. The decreased number of microvessels per microscopic field in both in situ and invasive carcinoma in comparison to hyperplastic areas suggests that this type of carcinoma once developed no longer requires a profuse vascular supply. The few vessels of the stroma of neoplastic lobular-alveolar structures are enough to sustain their relatively low rate of proliferation. By contrast, blood supply is a critical factor for most fast-growing transplantable tumors, even during their later stages. This necessity may account for IL-12's high efficacy against these tumors, even when they are large. With tumors that progress slowly, anti-angiogenic activity is only efficacious in specific progression stages. This narrow window of activity might account for the ineffectiveness of IL-12 in the management of human cancer, since only patients bearing advanced tumors are enrolled in clinical trials.

The anti-tumor action of IL-12 is not confined to its indirect influence on endothelial cells. Directly or through secondary cytokines it triggers lytic activity and mediator release in a variety of tumor-infiltrating leukocytes, thus offsetting the continuous generation of new transformed cells. The efficacy of IL-12 probably rests on the sum of its activities, and not simply on blocking of tumor neoangiogenensis, important as this may well be. In effect, further subdivision of the "Early" protocol into shorter treatment periods markedly reduced IL-12 efficacy. The lower efficacy of "Chronic" versus "Early" treatment could indicate that continuous IL-12 administration is suppressive, though this possibility is not endorsed by the results in FVB-neuN mice. It should be noted that from the second course mice of both strains received daily 100 ng/day IL-12 (i.e. around 4.5-7.7 μg/Kg). This dose is well tolerated and almost no-side effects appeared. It is probably close to the optimal active dose, since a ten or twenty-fold reduction abolishes its activity.

Cellular basis of the antiangiogenic effect of IL-12

In order to tease apart a few aspects of this composite IL-12 induced inhibition of neoangiogenesis and carcinogenesis progression, we set up the experimental system

shown in Fig. 2. The presence 0.1-100 ng/ml of IL-12 in the culture medium did not alter the expression of adhesion molecule, proliferative and migratory ability of the murine microvascular H.end endothelial cells (H.end). As these results apparently challenge many data on the effects of IL-12 on vascular system, we next investigated in the co-culture system of Fig. 2 whether the effect of IL-12 were mediated trough lymphoid cells.

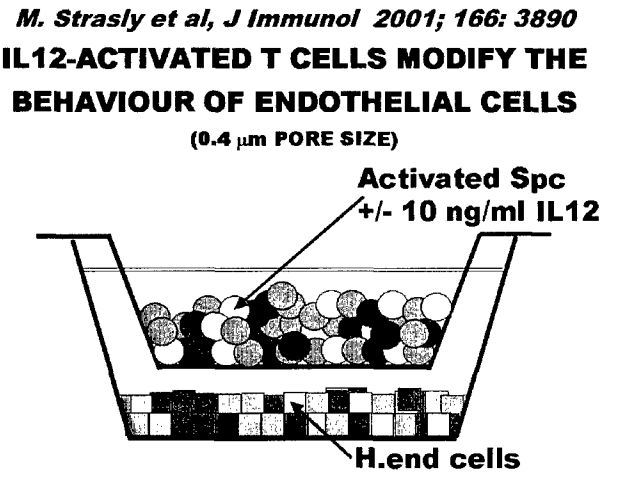

Fig. 2. *Transwell culture system. Activated spleen cells or spleen cells activated in the presence of IL-12 are cultured in the transwell insert. The effect of soluble factors released are evaluated on H.end endothelial cells growing at the bottom of the well.*

In this transwell system, the co-culture of lymphoid cells activated in the presence of IL-12 resulted in a dose-dependent inhibition of H.end cell proliferation. Moreover, the ability of H.end cells co-cultured with lymphoid cells activated in the presence of IL-12 to adhere to extracellular matrix was dramatically reduced. Factors released by lymphoid cells activated in the presence of IL-12 also increase the expression of VCAM-1 and ICAM-1, but not that of E-selectin on H end cells. To define the lymphoid cells population mostly responsible for the release of factors inhibiting H.end activities, IL-12 activated lymphoid cells from variously immunodeficient mice were used in the coculture. Lymphoid cells from *nu/nu* mice lacking T cell functions and displaying an

enhanced NK activity displayed a reduced ability to inhibit H.end activities, while no inhibition was afforded by lymphoid cells from NOD-scid mice exhibiting low NK activity and defective for macrophage function. Surprisingly, our data obtained with neutralizing antibody anti-IFN-γ or by using cells coming from IFNγ$^{-/-}$ knockout mice demonstrate that the lack of this cytokine did not completely abrogate the ability of activate lymphoid cells to inhibit H.end activities. This suggests that other molecules can substitute for IFNγ. The finding obtained in this study also suggest that an early circuit of soluble factors triggered by IL-12 requires a ping-pong of soluble signals between lymphoid and endothelial cells. The outcome of these interactions is the impairment of endothelial cells that become unable to sustain neoangiogenesis.

IL-12 modulation of tumor genetic programs

To further define the molecular basis of IL-12 inhibition of Her-2/neu mammary carcinogenesis, we exploited the same transwell culture system substituting the H.end culture at the bottom of the transwell with Her-2/neu carcinoma cells (TUBO cells). Lymphoid cells from normal and GKO mice were activated by anti-CD3 and anti-CD28 antibodies and cultured in inserts in the presence of IL12 or not (activated lymphocytes). Normal IL12-activated lymphocytes produced fivefold more IFN-γ, 20% less IL2 and equal amounts of IL4 than activated lymphocytes. Activated and IL-12 activated lymphocytes from GKO mice did not produce IFN-γ, whereas they released threefold more GM-CSF. The ability of activated lymphocytes co-cultured in transwell inserts to modulate the gene expression in Her-2/neu p185neu positive carcinoma cells (TUBO cells) growing at the bottom of wells was next evaluated. After 96 hours of coculture, modulation of gene expression was first assessed with the commercially available ATLAS mouse cDNA expression array from Clontech. With twofold expression as the cut-off threshold, TUBO cells co-cultured with activated lymphocytes from both normal and GKO mice up-modulated expression of the gene coding for the LPS receptor (CD 14; LPSR, SwissProt ACC: P10810). By contrast, TUBO cells co-cultured with normal IL12-activated lymphocytes up-modulated expression of LPSR and STAT1 (SwissProt ACC:

U06924), IRF-1 (SwissProt ACC: M21065) and IP-1 (SwissProt ACC: U19119). Expression of these three genes is regulated by IFN-γ secreted by IL12-activated lymphocytes.. By contrast, up-regulation of LPSR gene expression appeared to be independent of both the downstream secretion of IFN-γ and the presence of IL12. These data suggest that the factors released by activated and IL12-activated lymphocytes change the gene expression pattern in tumor cells. In order to overcome the rigidity imposed by the fixed cut-off chosen for evaluation of the macroarray data, the modulation of a few genes regulating key features of tumor-host immune relationship was probed by semi-quantitative RT-PCR. A marked over-expression of LMP2 and LMP7 genes as well as an increased expression of PA28 were found in TUBO cells co-cultured with activated lymphocytes from normal mice. The expression of these three immunoproteasome genes was further up-modulated when TUBO cells were co-cultured with normal IL12-activated lymphocytes while no up-modulation was found when they were co-cultured with activated lymphocytes and IL12-activated lymphocytes from GKO mice. Surprisingly, the up-modulated expression of IP-10 by TUBO cells co-cultured with activated and IL12-activated lymphocytes from GKO mice was not inhibited. This suggests that factors other than IFN-γ released by activated lymphoid cells regulate IP-10 gene expression. A similar IFN-γ-independent regulation takes place with the iNOS gene. It was up-regulated following tumor cell co-culture with activated Lymphocytes and further up-regulated by IL12-activated lymphocytes from normal mice. However, its up-regulation was also evident when TUBO cells were co-cultured with activated lymphocytes and IL12-activated lymphocytes from GKO mice. Expression of MCP1 is markedly up-modulated in TUBO and TSA cells co-cultured with normal IL12-activated lymphocytes and not in those co-cultured with GKO IL12-activated lymphocytes. It is also up-modulated in TSA cells co-cultured with activated lymphocytes. Expression of VEGF, a factor of crucial importance for tumor angiogenesis is markedly down-modulated in tumor cell lines co-cultured with both IL12-activated lymphocytes and to a lesser extent activated lymphocytes. Lastly, in TUBO cells co-cultured with IL12-activated lymphocytes only, expression of angiopoietin (Ang) 2 became evident. No down-modulation of VEGF gene

expression nor up-modulation of Ang2 gene expression was found when tumor cells were co-cultured with activated lymphocytes and IL12-activated lymphocytes from GKO mice.

In a blind fashion, two trained pathologists also evaluated whether the up-modulated expression of a few genes correlated with the over-expression of the proteins they encode, as assessed by immunocytochemistry. On culturing TUBO cells on microscope slides placed at the bottom of the transwells in co-cultures with activated lymphocytes and IL12-activated lymphocytes it was found that the up-modulation of MIG and IP-10 gene expression detected by semiquantitative RT-PCR went along with the intensity of protein expression. Immunocytochemistry data also endorse the indication of an IFN-γ-independent up-regulation of IP-10, but not of MIG expression when tumor cells are co-cultured with activated lymphocytes and IL12-activated lymphocytes from GKO mice. A similar activated and IL12-activated lymphocyte-dependent, but IFN-γ-independent up-regulation is also suggested for MIP-2, TGF and FGF protein expression. In the absence of IFN-γ, MIP-2 is much more strongly expressed by TSA cells. Lastly, VEGF is poorly expressed in tumor cells cultured with IL12-activated lymphocytes.

These data show that these Her-2/neu mammary carcinoma cells change gene and protein expression when co-cultured in the presence of activated T-lymphocytes and suggest a new way by which the immune system affects the growth of a tumor so that it becomes a party to its own inhibition.

Conclusion

The data obtained show that IL-12 delay the *neu* oncogene driven progression of mammary carcinogenesis chiefly by interfering with the passage from atypical hyperplasia to invasive carcinoma. This interference appears to mostly depend on indirect inhibition of tumor-associated angiogenesis. Its lower efficacy in more advanced lesions

and the dose range required pose some constraints on the use of IL-12 as an immunological alternative to current management of manifest neoplastic lesions. Nevertheless, the efficacy of IL-12 points to enhancement of nonspecific immunity as an effective way to prevent mammary tumors in individuals at risk. Lifetime administration is not required for genetically determined cancers with a long natural history, whereas definition of the carcinogenic events may enable the establishment of effective preventive treatments.

The prevention of carcinogenesis by delaying its progression is the result of multiple activities elicited by IL-12 in distinct cell populations. As reported in Fig. 3, IL-12 acts trough the induction of a first group of down stream cytokines (IFN-γ, TNF-α, and GM-CSF in special conditions). While these cytokines are active on many cells, their three main targets are: 1) endothelial cells; 2) lymphoid cells; 3) tumor cells.

A marked activation associated with inhibited proliferation in evident on tumor associated new formed endothelial cells. Lymphoid cells selectively recruited at tumor site by the adhesion molecules expressed by tumor associated vessels are variously activated by IL-12. Activated lymphoid cells and granulocytes inhibit tumor cell proliferation and highly damage tumor associated vessels. In addition, IL-12 induced downstream cytokines affect tumor genetic program by inhibiting tumor angiogenesis.

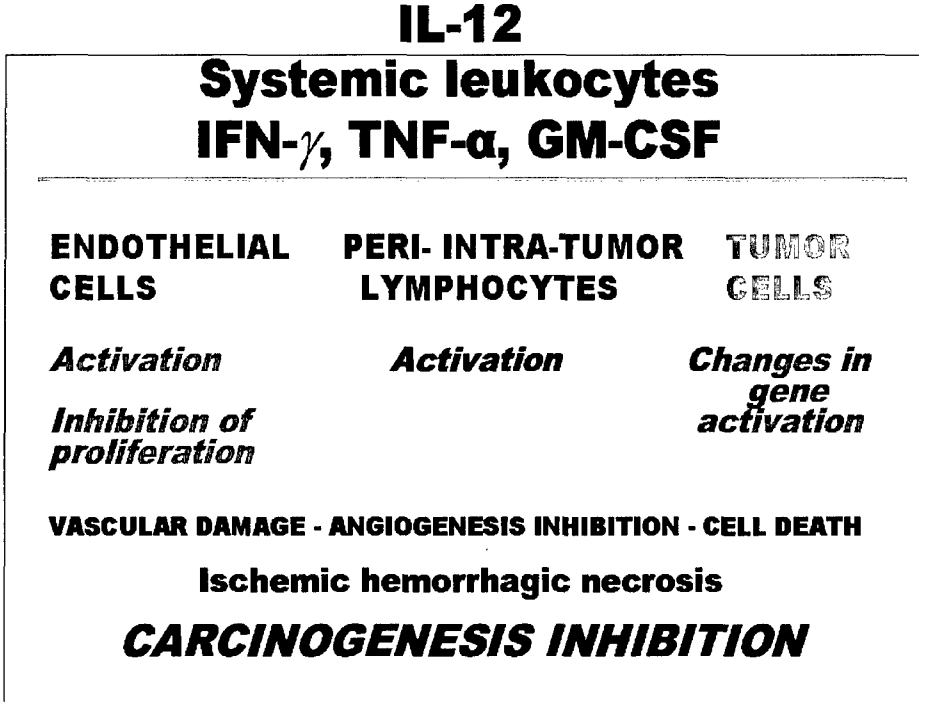

Fig. 3. *Interpretation of the events leading to IL-12 activated inhibition of He-2/neu carcinogenesis.*

TASK 2-3. PROPHYLACTIC VACCINATION WITH CYTOKINE GENE-TRANSDUCED TUMOR CELLS.

Goals

The aim of this task is prevent mammary carcinogenesis by eliciting a specific immunity to Her-2/neu p185 with cellular vaccines made of engineered tumor cells. To this end we established and characterized a number of new cell lines and clones derived from BALB-neuT (TUBO and AG12 cells) and FVB-neuN mice (N202.1A, N202.1E) that express high amounts of rat $p185^{neu}$ molecules on their cell membrane- These cells were also been used as recipient for transduction with genes coding for various cytokines (IFN-γ, IL-2, IL-12, IL-15). Previous studies by our group have that syngeneic tumor cells engineered to release cytokines are particularly effective in eliciting a strong antitumor

immunity (4). Moreover, allogeneic histocompatibility molecules (MHC class I) can significantly increase the immunogenicity of cellular vaccines engineered with cytokine genes.

Vaccination of BALB-neuT mice

The efficacy of allogeneneic N202.1A ($H2^q$) cells in eliciting a protective immunity in BALB-neuT ($H\text{-}2^d$) transgenic mice was first tested. These mice provide a very aggressive model of mammary carcinogenesis. Mice were vaccinated every 28 days (twice/week for two weeks) from the 6^{th} week of age with parental of cytokine gene engineered N202.1A cells. As shown in Table 3, no inhibition of carcinogenesis was observed in mice vaccinated every 28days with rat p185neu positive allogeneic cells. By contrast a significant protection was afforded by the vaccination with N202.1A cells engineered to release cytokines. Apparently the best protection was provided by N202.1A cells engineered to release IL-12.

Table III. Prevention of mammary carcinomas in BALB-neuT mice trough vaccination with allogeneic cells engineered to release cytokines

Cell vaccine	Gene transfection	Tumor-free mice At 30 weeks of age	%
None	None	0/8	0%
N202.1A	None	0/7	0%
N202.1A	IFN–γ	4/8	50%
N202.1A	IL-2	3/8	38%
N202.1A	IL-12	8/8	100%
N202.1A	IL-15	2/8	25%

To further increase the efficacy, cellular vaccines were combined with the chronic IL-12 treatment of mice outlined under Task 1. In this way a series of multicomponent

vaccination treatments were evaluated. A first set of experiments with allogeneic cells alone and in combination with IL-12 is finished, and the manuscript submitted. Other experiments are still more preliminary, particularly due to the length o the in vivo observation. At least 52 weeks are required to assess the preventive potential of the treatment.

Allogeneic Her-2/neu positive cells and IL-12

In a first set of experiments 6 weeks old BALB-neuT mice were vaccinated twice-weekly with parental, non engineered N202.1A allogeneic mammary carcinoma cells expressing high levels of both $p185^{neu}$ protein and $H\text{-}2^{q}$ class I molecules. followed by five daily administrations of 100 ng of IL-12. After one week of rest this 3 week course was repeated until mice were sacrificed or reached the age of one year. Mice receiving this combined treatment were referred to as "treated mice". This combined treatment very effectively inhibited the development of mammary carcinomas. All control mice developed their first mammary tumor within 22 weeks, whereas all treated mice were completely tumor-free. Within 30 weeks all control mice developed tumors in all ten mammary glands. At 52 weeks (end of the study) 88% of treated mice were still completely tumor free. The lifetime of treated mice was more than doubled.

The relative contribution of the components of this protective treatment was assessed by their single administration. IL-12 alone produced a significant delay in tumor latency and a significant reduction in tumor multiplicity, but did not affect tumor incidence, in agreement with our previous findings (see above). Vaccination with Neu/$H\text{-}2^{q}$ cells alone delayed tumor onset, but did not result in tumor-free mice, thus indicating that IL-12 markedly contributes to the efficacy of the combined treatment. However, since vaccination with Neu/$H\text{-}2^{q}$ cells alone delayed tumor onset whereas that with the $Neu^{neg}/H\text{-}2^{q}$ cell variant was ineffective, also $p185^{neu}$ recognition appears to plays a significant role. The need for live and replicating cells in the vaccine was also evaluated since block of cell proliferation by irradiation or mitomycin C may reduce the immunogenicity of a vaccine, while vaccination with replicating cells poses practical

issues. When mitomycin C treated Neu/H-2^q cells were used in combination with IL-12, carcinogenesis was blocked and all mice were tumor-free at one year. Vaccination with mitomycin C-blocked cells allowed us to evaluate also the efficacy of syngeneic Neu/H-2^d cells, which otherwise would be tumorigenic: no protection was obtained with such cells alone, whereas delay in tumor occurrence induced by Neu/H-2^d cells and IL-12 was not longer of that caused by IL-12 alone. These data suggest that allogeneic MHC molecules are required for an effective treatment.

Pathological evolution of the mammary gland.

By the 3rd week of age foci of atypical hyperplasia were evident in the terminal ductal lobular units (TDLU) of control mice and subsequently extended to all ten mammary glands. Foci of carcinoma *in situ* first apparent around the 15th week evolved to invasive lobular carcinomas by the 20th. Ten weeks later invasive lobular carcinomas were present in all the glands. Treatment with IL-12 alone slowed down this progression. The atypical foci grew much more slowly and the onset of *in situ* and invasive carcinomas was delayed by 5-6 weeks. The lesions were invaded by reactive cells, whereas a similar infiltrate was barely visible in the controls. At the 15th week of age the TDLU of treated mice were carpeted with a single layer of epithelial cells and surrounded by reactive cells. The few small hyperplastic foci were the scene of intense inflammation . After the fifth course (at 27 weeks), the mammary tissue was mainly composed of small ducts with few TDLU. Remaining TDLU were slightly hyperplastic and surrounded by reactive cells. These were sometimes found inside the lobular structures among the epithelial cells. In treated mice, attenuated inflammation was present at 35, 40 and 45 weeks of age after the eighth or tenth course. In very few mice only invasive lobular carcinomas developing from hyperplastic foci were microscopically detectable. Their lobules were less compact than in the controls, while the neoplastic epithelial cells were poorly coherent and frequently disaggregated, and gave rise to fissures. The stroma inside and around the tumor was more abundant and occupied by a distinct inflammatory infiltrate. Reactive cells were numerous in the stroma of both the hyperplastic and the tumor lesions, and occasionally intermingled with the neoplastic cells after crossing or

damaging the basal membrane. Immunohistochemical examination after the second course (15th week of age) revealed a reduction of hyperplastic foci and TDLU containing epithelial cells expressing p185^{neu}. At 27 weeks, the mammary tissue of treated mice was constituted only by small ducts lined by a single layer of epithelial cells without or with a slight p185^{neu} expression confined to the cytoplasm associated with a poor positivity to proliferating cell nuclear antigen (PCNA). By contrast, a marked cytoplasmic and membrane p185^{neu} expression similar to that of hyperplastic and neoplastic lesions from untreated and IL-12 treated mice was displayed by the few carcinomas detected in mice treated with IL-12 and cell vaccine. A conspicuous macrophage, neutrophil and CD8$^+$ lymphocyte infiltrate was present in the hyperplastic foci and in the tumor stroma of the treated mice. The number of dendritic and NK cells also increased, though it was not higher than in mice treated with IL-12 only. Recruitment of reactive cells was accompanied by overexpression of endothelial adhesion molecules in the small vessels. Significantly fewer blood vessels were found in mammary glands of treated mice and of those receiving IL-12 only than in the controls. By contrast, proinflammatory cytokines and chemokines were more evident in treated mice than in mice receiving IL-12 only and were almost absent in mammary glands from untreated controls.

Cell-mediated reactivity of treated mice.

At week 15 after the second course, groups of 3-5 control and treated mice were sacrificed to evaluate spleen cell reactivity. Both total spleen cell yield and the absolute number of CD4$^+$ cells were significantly higher in mice treated with Neu/H-2^q cells and IL-12. However, repeated CTL assays against HER-2/neu-positive or negative targets showed only about 10% of specific lysis of HER-2/neu$^+$ targets by spleen cells from BALB-neuT mice vaccinated with Neu/H-2^q cells and IL-12. These spleen cells did not display any protective activity when they were admixed with a tumorigenic dose of Neu/H-2^d syngeneic mammary tumor cells in a Winn-type neutralization assay in BALB-neuT mice (data not shown). On the other hand, an increased spontaneous proliferation was displayed *in vitro* by the CD4$^+$ lymphocyte subpopulation. It was further increased in an antigen-specific manner by the addition of mitomycin blocked Neu/H-2^q cells.

Moreover, total spleen cells and $CD8^+$ lymphocytes of mice treated with Neu/H-2^q cells and IL-12 spontaneously released more IFN-γ than leukocytes from control or IL-12 treated mice. Large amounts of IFN-γ were released by total and $CD8^+$ spleen cells following restimulation by Neu/H-2^q cells. IL-4 was secreted spontaneously or following restimulation by both total spleen cells and $CD4^+$ lymphocytes (but not by $CD8^+$ lymphocytes) from mice treated with Neu/H-2^q cells and IL-12. This cytokine release is mostly triggered by the recognition of H-2^q allogeneic MHC glycoproteins since similar amounts of IFN-γ and IL-4 are released following restimulation with Neu/H-2^q and Neu^{neg}/H-2^q cells.

Allogeneic Her-2/neu positive cells engineered to release IFN-γ *and systemic IL-12*

Four distinct signals were combined in these experiments: $p185^{neu}$, IFN-γ, allogeneic MHC class I antigens, and IL-12. The onset of mammary carcinomas was strongly inhibited in transgenic mice receiving this type of treatment and 88% of vaccinated mice are alive and tumor-free at 52 weeks of age, as opposed to 0% of MSA (murine serum albumin) treated controls. In this combination, too, when the weight of the elements required to produce a significant immunity from mammary carcinoma growth were dissected it was found that both the specific vaccine and the nonspecific stimuli such as IL-12 and allogeneic MHC antigens were required to produce a maximal effect.

The increase in immunogenicity gained by tumor cells engineered to release IFN-γ is limited to the first 24-30 week as compared to the wild type N202.1A allogeneic cells. We are currently comparing the protective ability of the allogeneic cells engineered to release different cytokines. The aspect that appears to as the most interesting is to evaluated whether the release of cytokine made the vaccine effective in protecting mice with more advanced preneoplastic lesions.

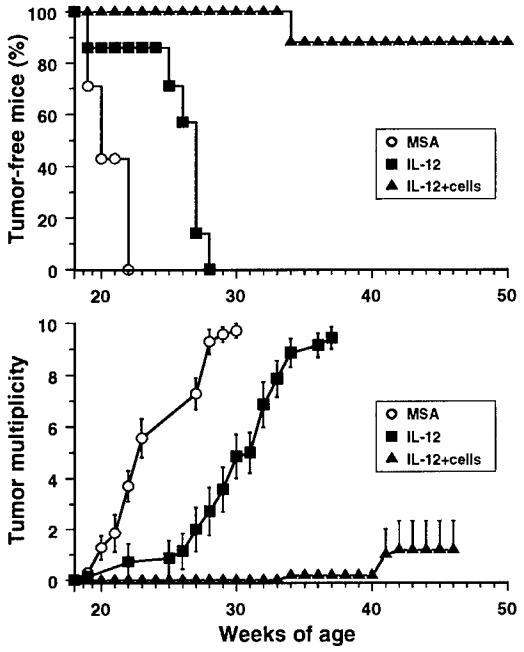

Fig. 4. *Comparison of IL-12 alone and IL-12 + cellular vaccine in the protection of BALB-neuT mice from the development of mammary carcinoma.*

Conclusions

The data obtained fulfill the goals of this specific task. Combination of an allogeneic cell vaccine and IL-12 prevented the onset of mammary carcinoma in tumor-prone BALB-neuT mice. The lifespan of treated mice was more than doubled, while the quality of their life was not impaired. The treatment combined three of four immunological stimuli, namely 1) $p185^{neu}$; 2) allogeneic class I MHC glycoproteins; 3) IL-12; 4) in a few cases additional cytokines released by engineered allogeneic cells. The comparison between effects obtained with syngeneic Neu/H-2^d and allogeneic Neu/H-2^q cells shows the weight of allogeneic MHC signal, whereas the comparison

Table IV. Analysis of individual components of the multicomponent vaccine.

Cell vaccine			IL12	Tumor-free mice at	
Neu	Allo-MHC	IFN-γ gene		24wk of age	46 wk of age
-	-	-	-	0%	0%
+	+	+	+	100%	88%
+	+	+	-	88%	12%
+	+	-	+	88%	88%
+	+	-	-	63%	12%
-	-	-	+	86%	0%
-	+	-	-	0%	0%

between Neu/H-2^q and Neu^{neg}/H-2^q cells underlines the importance of $p185^{neu}$ recognition.

Evaluation of the mechanisms mediating tumor prevention revealed the activation of multiple immune responses. The loss of efficacy of this combined treatment in IFN-γ gene knockout transgenic mice depicts the central role played by IFN-γ in all the mechanisms involved in carcinogenesis prevention. IL-12 alone significantly delays tumor progression through its effects on nonspecific cell-mediated immunity and angiogenesis. Tumors arising in BALB-neuT mice receiving IL-12 alone were infiltrated by dendritic cells, NK cells and $CD8^+$ lymphocytes. The local release of IFN-γ induced the expression of tertiary antiangiogenic mediators such as IP-10 and MIG, and the microvessel density was decreased. The combination of a cellular vaccine with IL-12 elicited new responses and enhanced effects produced of IL-12. The cell-mediated inflammatory response was conspicuous in the mammary glands of mice receiving the

combined treatment. Production of MIP-2 and the expression of endothelial adhesion molecules correlated with the recruitment of granulocytes, which appear to play an important role through the damage they cause to the tumor vessels and their role in antibody-dependent cytotoxicity.

The combined treatment, but not IL-12 alone, elicited a marked specific humoral immune response. Anti-$p185^{neu}$ antibodies in the sera of treated mice may impair carcinogenesis by inducing a functional block of $p185^{neu}$ receptor function, down-regulating its expression on the cell membrane, and impeding its ability to form the homo- or heterodimers that spontaneously transduce proliferative signals to the cells. In control mice actively proliferating $p185^{neu+}$ cells were evident in the TDLU from the 3rd week onwards and gave rise to hyperplasia first and then to carcinoma. By contrast, the reduced number of $p185^{neu}$ positive cells, the intracytoplasmic confinement of $p185^{neu}$ and the diminished nuclear positivity to anti- PCNA mAb in the mammary glands of treated mice after the second course point to a direct inhibitory activity by anti-$p185^{neu}$ antibody. The mammary glands of treated mice displayed fewer and fewer TDLU after each course. This is a critical issue in tumor prevention since progression leading to the formation of invasive lobular carcinomas origins from TDLU proliferating cells (PCNA positive) that highly expresses $p185^{neu}$. This TDLU-limited autoimmunity associated with the restraint of the $p185^{neu}$ function may depend on both the anti-$p185^{neu}$ antibodies and on the specific responses elicited in treated mice.

Activation of $CD4^+$ and $CD8^+$ cells was associated with cytokine release within the mammary gland and in the spleen. Moreover, infiltrating lymphocytes were present in the stroma surrounding neoplastic cells and penetrated the basal membrane to interact with $p185^{neu+}$ neoplastic epithelial cells.

In human carcinomas and melanoma, the presence of a reactive infiltrate intermingled with tumor cells, as opposed to the presence of leukocytes at the periphery of tumor nests, often correlates with a more favorable prognosis (Fig 5).

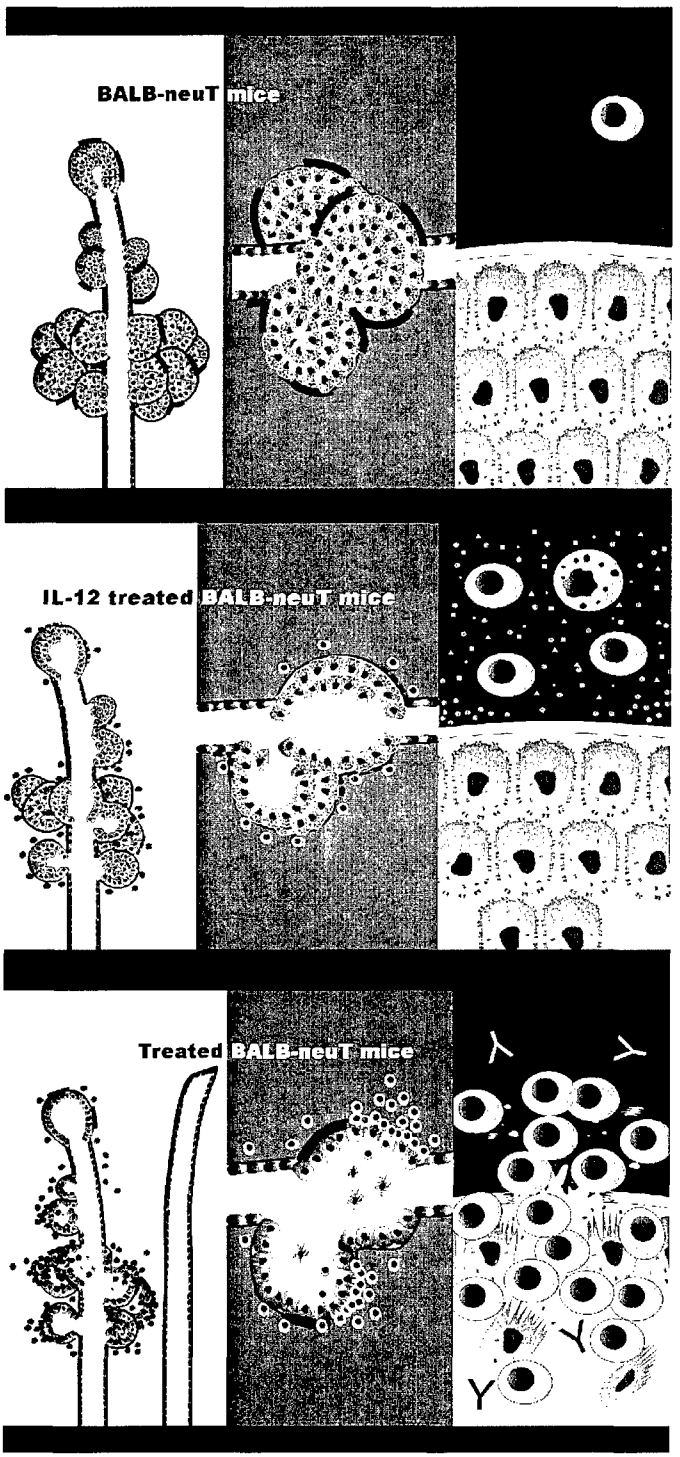

Fig. 5. *Upper panel: The ductules of mammary gland of normal mice end with terminal buds. During carcinogenensisi in BALB-neuT mice there is the formation of lateral buds. These cells express p185^{neu} (orange). A scarce reactive infiltrate is evident around the mammary gland. In IL-12 treated BALN-neu mice (Middle panel) secondary*

and tertiary cytokines and chemokines induced by IL-12 activate lymphocytes and impair tumor angiogenesis. This reaction markedly delay the progression of the neoplastic lesion, while does not affect the expression of p185^{neu}. A marked infiltration of reactive lymphoid cells, production of antibody and destruction of p185^{neu} positive tumor cells take place in mice vaccinated with allogeneic cells and treated with systemic IL-12 (Lower panel)

This aggressive infiltration was not observed in BALB-neuT receiving IL-12 alone or Neu/H-2^{q} cells alone. The activation of T cell reactivity appears to be a feature associated with the combined treatment and not to the presence of p185^{neu} alone. The simultaneous membrane expression of p185^{neu} and allogeneic H-2^{q} MHC glycoproteins and systemic IL-12 are instrumental to overcome T cell tolerance against the transgenic rat p185^{neu}, a xenogeneic protein that shares more than 94% homology with mouse p185^{neu} already markedly expressed in TDLU of weaning BALB-neuT mice. In vitro no evidence of a major involvement of specific CTL activity was found. Even though the possibility that CTL activity could contribute to the preventive effect cannot be ruled out, present data show that in vitro cytotoxicity and Winn-type tumor neutralization assay are not predictive of treatment efficacy. By contrast, the inefficacy of the combined treatment in BALB-neuT/GKO mice suggests that different mechanisms activated by IL-12, MHC alloreactivity and p185^{neu} recognition include a crucial step mediated by IFN-γ, a multifaceted regulator of immune activity. However, other properties of IFN-γ could play an important role in the prevention of carcinogenesis. IFN-γ inhibits the proliferation, the angiogenic phenotype and the HER-2/neu-mediated signaling pathways of p185^{neu} cells.

An appropriate combination of specific and non specific immune stimulation set in motion distinct defense mechanisms. Each component of the treatment plays a own role and complete protection is obtained when all components were included. Their concerted action stopped a devastating cancer-prone condition affecting all the ten mammary glands, and more than doubled the lifespan of BALB-neuT mice. This impressive result suggests that a similar approach may prevent tumors in persons with a high risk of

cancer. Due to the complexity and duration of treatment, the relative role of specific and nonspecific mechanisms is still hardly identifiable. The breeding of BALB-neuT mice selectively deficient for specific immune functions will probably allow to better tease apart the weight of each immune mechanisms involved.

TASK 4. VACCINATION WITH HER-2/*neu* PEPTIDES

Goals

While the Task 1 and Task 2 provided positive suggestions on the a possible immunological control of Her-2/neu angiogenesis, the data obtained studying peptide vaccination were mostly negative. The specific aim is to prevent mammary carcinoma by vaccination with peptides derived from the Her-2/*neu* oncogene. Since the binding motifs of the H-2^d haplotype are well known (while H-2^q has not been thoroughly studied) we have studied this approach in BALB-neuT mice, of H-2^d background.

Peptides and their immunogenicity

The first step was to identify peptide sequences in the neu gene that bind to H-2^d class I gene products leading to receptor-mediated recognition by T lymphocytes. We derived the peptide motifs for binding in the grooves of H-$2D^d$, H-$2K^d$, H-$2L^d$. The chosen peptides derive all from the intracellular domain of the $p185^{neu}$. They are designated: P114-003 (amino acids 249-257), selected for binding to H-$2D^d$; P114-002 (amino acids 558-566), selected for binding to H-$2K^d$; and P114-004 (amino acids 66-76). This 11 amino acid long peptide binds to both H-$2L^d$ and H-$2D^d$. All the peptides were synthesized by Primm Srl (Milan, Italy).

Peptide sequences

p 66-76	(# 4)	T Y V P A N A S L S F	H2-K (p66-74), H2-L (p68-76)
p 249-257	(# 3)	T G P K H S D C L	H2-D
p 444-452	(# 1)	A Y S L T L Q G	H2-K
p 558-566	(# 2)	E Y V S D K R C	H2-K

Fig.6. *The first series of selected Her-2/neu peptides*

The second step was to assess the immunogenicity of the three peptides and analyze the immune response by using short-term vaccination protocols and in vitro cell-mediated cytotoxicity assays. First, immunogenicity was evaluated in BALB/c mice (H-2^d), where the rat $p185^{neu}$ protein is an exogenous antigen with marked homology with autologous mouse $p185^{neu}$ protein. Mice were immunized intradermally, once a week for 4 weeks, with 100 μg of peptide in 50 μl of PBS. One week after the last immunization, mice were challenged subcutaneously with a cell line derived from a BALB-neuT carcinoma (TUBO cells) and observed for tumor appearance and progression. No differences were observed between control and peptide-vaccinated mice (data not shown). In other experiments, mice received 1 x 10^7 syngeneic spleen cells (Spc) pulsed overnight with peptides. One week after the last immunization, a few mice were sacrificed to asses Spc cytotoxity against TUBO cells after six days in vitro restimulation with mitomycin-C treated (Sigma, St. Louis, MO; 100 μg/10^7 cells/ml for 30 min) TUBO cells. Cytotoxic activity was expressed as Lytic $Units_{20}$ (LU). Other mice were challenged subcutaneously with TUBO cells and observed for tumor appearance and progression. Our data show that Spc from these vaccinated mice are able to prime for a TUBO specific cytotoxic response, as shown by in vitro cytotoxicity assays (Table 3).

Table V. Cytotoxicity against TUBO cells of Spc from vaccinated mice after in vitro stimulation with Mitomycin-C treated TUBO cells.

Mice immunized with:	Cytotoxicity (LU ± SD)
None	42 ± 7
Spc not pulsed	193 ± 12
Spc pulsed with P114-002	246 ± 41
Spc pulsed with P114-003	302 ± 14
Spc pulsed with P114-004	227 ± 18

Despite this feeble reactivity, Spc from immunized mice do not inhibit the in vivo growth of TUBO cells (Table 4).

Table VI. Effect of vaccination with Spc pulsed with peptides on the in vivo growth of TUBO cells in BALB/c mice.

Mice immunized with:	Tumor take	Latency time[a]	Survival time[b]
None	5/5	24 ± 1	48 ± 8
Spc not pulsed	5/5	24 ± 2	49 ± 2
Spc pulsed with P114-002	5/5	26 ± 2	54 ± 7
Spc pulsed with P114-003	5/5	25 ± 1	51 ± 3
Spc pulsed with P114-004	5/5	26 ± 1	48 ± 9

[a]Latency time: time in days between the challenge and the appearance of tumors > 3 mm mean diameter.
[b]Survival time: time in days between the challenge and the appearance of tumors > 10 mm mean diameter.

The third step was the actual vaccination of BALB-neuT mice in which rat $p185^{neu}$ is a fully tolerated self antigen. Starting from the seventh week of age, mice were immunized with 10^7 syngeneic Spc pulsed with peptides, once a week for four weeks, followed by three weeks off. This course was repeated four times. As shown in Fig.7, no significant differences were observed as far as the mean number of tumors per mouse was considered. However, mice vaccinated with Spc pulsed with P114-004 displayed lower values.

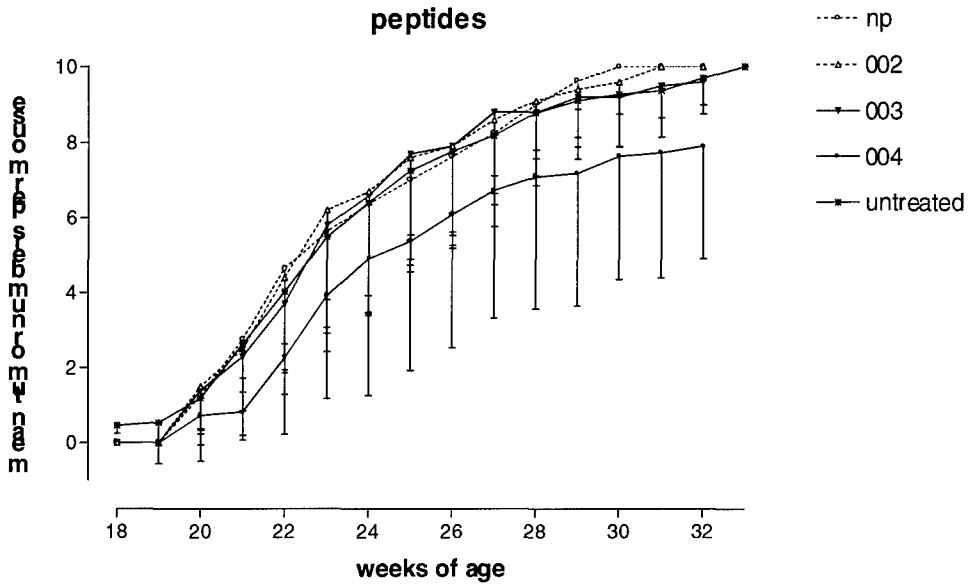

Figure 7. *Effect of vaccination with Spc not pulsed (np) or pulsed with the various peptides (002, 003, 004) on the development of spontaneous mammary carcinomas in BALB-neuT mice. Each group: 10 mice*

Similar results were obtained with a new series of peptides, modified to better fit in the grooves of $H\text{-}2D^d$, $H\text{-}2K^d$, $H\text{-}2L^d$. (Fig. 8).

Modified peptides

p 66-76	E Y V P A N A K L S F	E66 K73
p 249-257	T G P K H S D C L	
p 444-452	E Y S L T K Q G L	E444 K449
p 558-566	E Y V S D K R C L	

Fig. 8. *The second series of Her-2/neu peptides. In blue the changed residues*

Lastly, much longer peptides were selected. BALB-neuT mice were immunized with these peptides either emusified with Freund's incomplete adjuvant or with peptides linked to KLH (Fig 9). All variously immunized BALB-neuT mice develop tumors in all mammary glands, even if in a few groups the kinetics of tumor appearance was delayed of about one week.

Conclusions

Admittedly we were unable to elicit a response able to delay Her-2/neu carcinogenesis in BALB-neuT mice by peptide vaccination. Nor the identification of a minimal immunogenic epitope was obtained. It is possible that the peptides alone or admixed with adjuvants were not able to break tolerance to rat Her-2/neu of BALB-neuT mice. $p185^{neu}$ is an antigen markedly expressed in the rudiments of the mammary gland of 3-week-old BALB-neuT female mice (Fig.1).

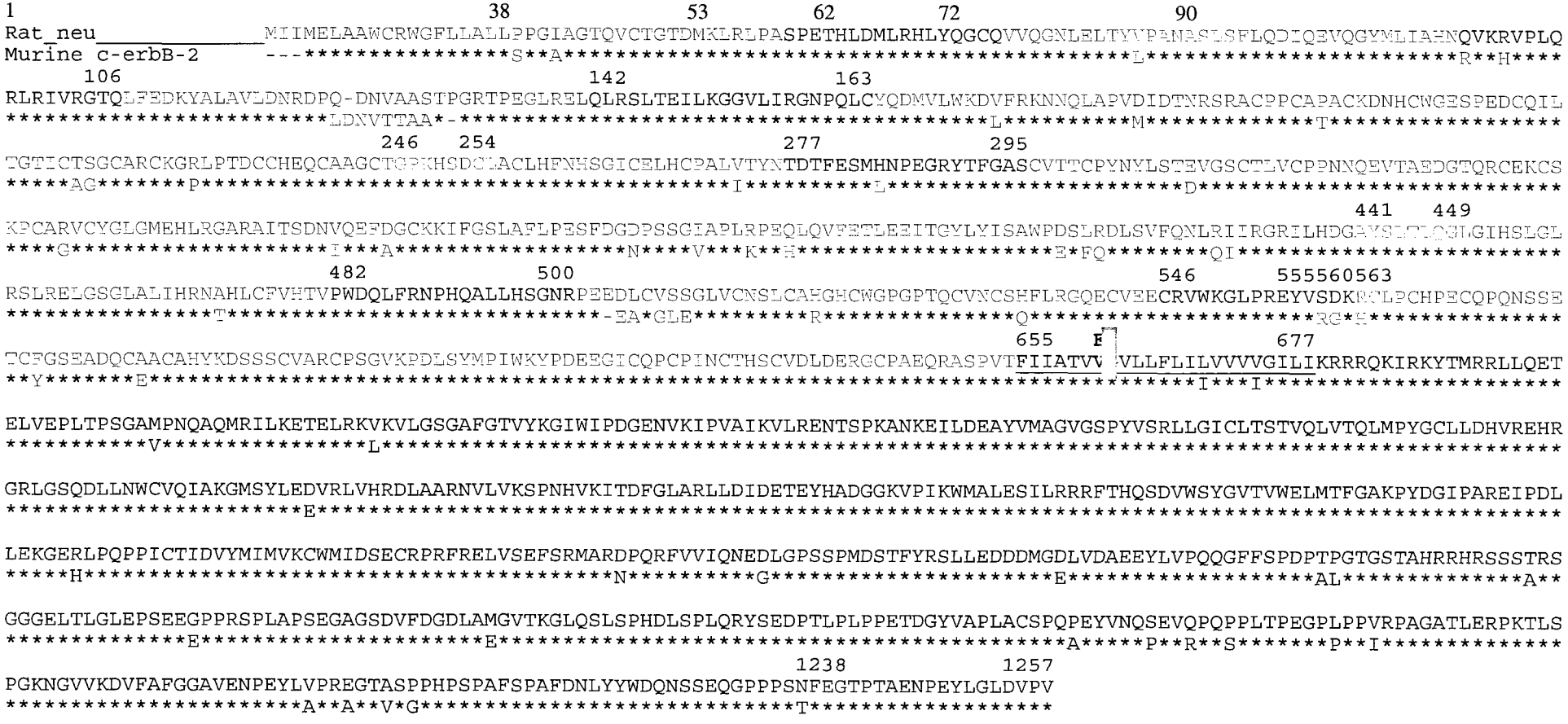

Fig.9. *The third series of selected Her-2/neu peptides. The whole rat and mouse p185neu amino acid sequence is shown. Green: The amino acid residues of the extracellular domain; Red: The amino acid residues of the transmembrane domain; Blue: The amino acid residues of the intracytoplasmatic domain. The peptides of the first and second selection are shown in yellow boxes while the mutation of the activated oncogene in light blue. Purple: The amino acid residues of the longer peptides of the third series.*

It is also possible that conformational and not linear determinant are of critical importance. Even the longer peptides of the third generation were not able to give rise to conformational determinants.

Very good protection was afforded to BALKB-neuT mice by DNA immunization by using various plasmids coding for the extracellular or the extracellular and transmembrane domain of p185neu (10, 11, 12). The striking contrast between the impressive protection observed after immunization with these plasmids or allogeneic whole cells and the poor results obtained with Her-2/neu peptides lead us to gloomy abandon the peptide immunization. Potentially it was a very informative approach that should have permitted the identification of a few crucial epitopes of the target $p185^{neu}$ molecule.

TASK 5. STUDIES IN IMMUNODEPRESSED MICE

Goals

The specific aim of this task is to define the immune mechanisms that play a major role in the inhibition of Her-2/neu mammary carcinogenesis. A thorough assessment of the immunological mechanisms leading to the delay of carcinogenesis progression following IL-12 administration is now reported in great detail in the sections *"IL-12 inhibition of angiogenic switch"*, *"Cellular basis of the antiangiogenic effect of IL-12"*, and *"IL-12 modulation of tumor genetic programs"* of this Final report, and in the "Appendice" manuscripts. Moreover in the sections *"Allogeneic Her-2/neu positive cells and IL-12"*, *"Pathological evolution of the mammary gland"*, and *"Cell-mediated reactivity of treated mice"* the immunological mechanisms leading to the almost complete inhibition of Her-2/neu carcinogenesis following allogeneic cell vaccination have been analyzed in detail. Less information was acquired on the mechanisms elicited after peptide vaccination in consideration of the poor functional results we have observed in these experiments.

As planned in the original proposal, these immunological data have been acquired in the last phases of this research project, when the effectiveness of the distinct treatments was evident. Serum IFN-γ levels have been monitored in treated mice and *in vitro* tests, while cell mediated cytotoxicity, cytokine production, gene expression and immunohistochemical analysis were used to define the reactive mechanisms elicited by the various treatments.

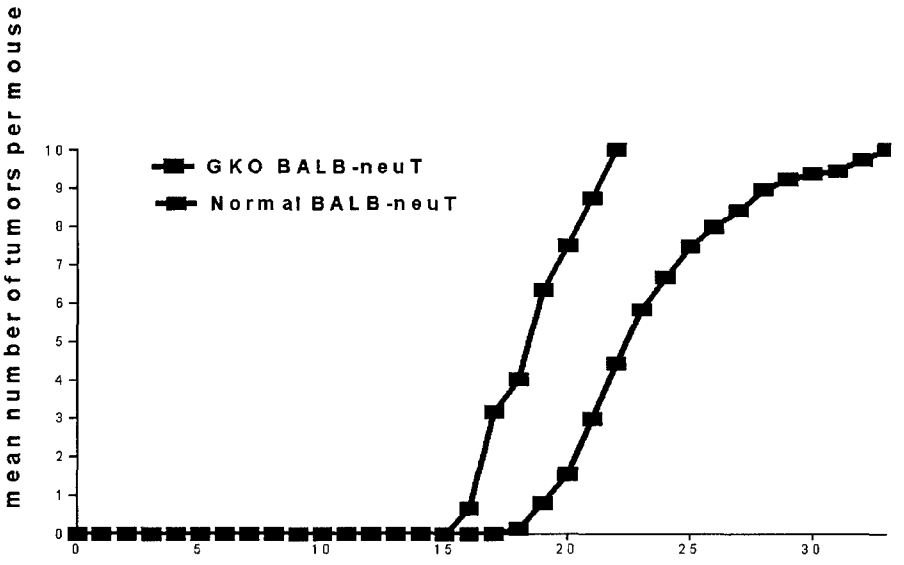

Fig. 10. *Progression of mammary carcinogenesis in BALB-neuT (purple) and BALB-neuT/GKO mice. Each group consist of 10 mice*

More recently we have studied the progression of carcinogenesis and the efficacy of various treatments by crossing GKO BALB/c mice with BALB-neuT mice. In these

BALB-neuT/GKO mice Her-2 neu carcinogenesis is faster (Fig 10) while tumor associated vessels are larger and more numerous.

The combined administration of Neu/H-2^q cells plus IL-12 is ineffective in BALB-neuT/GKO mice. This fading of the protection points to a crucial role of IFN-γ in the prevention of Her-2/neu carcinogenesis. IFN-γ inhibits the proliferation of Neu/H-2^d cells, down modulates their membrane expression of $p185^{neu}$ and the production of the proangiogenic protease MMP9, while it strongly up modulates the expression of class I H-2 glycoprotein and the production of antiangiogenic chemokines such as IP-10 and MIG.

7. KEY RESEARCH ACCOMPLISHMENTS

- Found that carcinogenesis is an appropriate and rational goal for immunological attack. The slow progression of carcinogenesis makes it more sensitive to immune mechanisms than fast growing transplantable tumors

- Found that repeated administrations of IL-12 effectively reduce tumor incidence and delay time of appearance of Her-2/neu tumors that develop in genetically predisposed mice

- Found that the above inhibition can be obtained with low doses of IL-12 administered during the angiogenic switch that accompanies the passage from preneoplastic lesions to invasive stage of Her-2/neu carcinogenesis

- Found that IL-12 induced downstream mediators change the genetic program of carcinoma cells and elicit antiangiogenic activities in tumor cells

- Found that specific vaccination with $p185^{neu}$ expressed by allogeneic carcinoma cells alone, engineered to release IFN-γ, or coded by DNA plasmids effectively and persistently inhibit Her-2neu carcinogenesis

- Found that the combination of specific anti p185neu vaccination combined with systemic IL-12 administration provide a long-lasting protection

8. REPORTABLE OUTCOMES

Research
Manuscripts

- Lollini P-L and Forni G., 1999. Specific and non-specific immunity in the prevention of spontaneous tumors. *Immunol. Today* 20, 343-350.

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9. CONCLUSIONS

Our results show that the carcinogenic process giving rise to mammary carcinoma in Her-2/neu transgenic mice can be effectively prevented by immunological approaches. IL-12 stimulation of nonspecific immunity delay the progression of the mammary carcinogenesis. IL-12 elicits a multi-factorial immune reaction resulting in a major inhibition of tumor associate carcinogenesis. Tumor prevention results from a delay of carcinogenesis progression. Potentially all mice will eventually die because tumor outgrowth. However, the delay may exceed the normal life span of a mouse. So, an effective protection is thus afforded in the absence of any characterization of target antigens.

A much stronger inhibition of carcinogenesis can be obtained combining specific and nonspecific immune stimuli that included a vaccine based on the recognition of epitopes of $p185^{neu}$ protein presented by allogeneic tumor cells in association with systemic IL-12 administration. In this case prevention rests on the destruction of the cells expressing target p185neu target protein. This combined specific and nonspecific treatment

appears to definitively save the mice from the multiple mammary carcinomas to which they are genetically predestined.

So what? The application of similar "soft" immunological preventive approaches to woman at risk could lead to significant improvements in the prevention of mammary carcinoma. Present experimental data may be enough to justify a fresh immunological management of the risk of cancer in clinics.

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Specific and nonspecific immunity in the prevention of spontaneous tumours

Pier-Luigi Lollini and Guido Forni

Antitumour vaccines cannot cure established tumours. However, tumour cells engineered to release cytokines can halt progression to cancer by inhibiting the angiogenic phenotype of pretumoural cells and activating tumour-associated leukocytes, thus they might prove useful in immunizing those at risk of cancer.

The presentation of tumour-associated antigens by dendritic cells demonstrates that defined peptides can elicit a specific antitumour immunity[1]. The recognition of multiple dominant and subdominant tumour antigens by the immune system can be evoked by whole tumour cells engineered with adhesion and costimulatory molecules, suicide genes or cytokines (reviewed in Ref. 2). When a tumour is engineered to release cytokines, it is the type of cytokine released that decides which immune mechanisms are elicited in a privileged way. Thus, selective activation of cytokines that are most appropriate to the stages of tumour progression is feasible[3].

These vaccine strategies are often effective. Tumours that in more conventional ways are unable to induce significant immune responses, evoke them when their antigens are presented by dendritic cells[1,2] or their engineered cells are used for vaccination[2,3]. This has altered our approach to the immunoregulation of tumours. However, the usual pitfall in evaluating the potential of these new vaccines is to overlook the fact that abrogation of the tumourigenicity of gene-engineered cells, and effective immunization of healthy animals against subsequent challenge by wild-type tumour cells, have little to do with therapy[4]. Their real ability to cure existing tumours has hardly ever been investigated. In most studies, only a minority of tumour-bearing mice were cured, and this limited efficacy was solely achieved when the vaccine was administered in the first few days after challenge[5]; corresponding clinical trials have not disclosed any significant ability to cure[6].

Nonetheless, further consideration must be given to the inability of these vaccines to prevent tumours. It may well be that their failure is too bitter a notion to swallow and hence is either consciously or unconsciously ignored. Several objectives have been made possible by these vaccines (Box 1), and the cure of clinically evident tumours has been the most implausible and, at the same time, the most common goal in clinical trials. The many ethical and emotional issues raised by cancer provide the main reason for these clinical attempts. Paradoxically, their failure is often blamed on the poor reliability of results from mouse models. In addition, more efficacious vaccines are called for. Experimental models suggest that vaccines engineered with combinations of genes are more effective than those with one only. However, to date, there is no clinical evidence to support this view[6].

Here, we emphasize that the high degree of immunogenicity that these new vaccines can offer is 'far too weighty a baby to be simply thrown out with the bathwater'. Improved vaccination is perhaps less important than the realization that the mechanisms elicited by specific immunization are not suited to cure established tumours. This issue is underscored, first, by the demonstration that these mechanisms lead to the rejection of normal tissues, but not to the rejection of tumours expressing the same target antigen[7]; and second, by the many ways

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Appendix 1

Box 1. What can be gained by interfering with the tumour–host immune relationship?

- Induction (or increase) of surveillance against tumour onset
- Induction of resistance to minimal residual disease and tumour recurrences
- Restraining the progression of clinically evident tumours
- Successful treatment of clinically diagnosed tumours

in which an established tumour manages to elude these immune mechanisms [F. Cavallo, (1997) *Immunological Blackboard* (Vol. 1, No. 1) http://pages.inrete.it/immunoblack]

Vaccination is a distinct example of preventive medicine, whereas 'therapeutic vaccination' is a distorted concept that has had no great success, even in the handling of infectious diseases. Despite this, the experimental data indicate that cancer vaccines should be able to cope with minimal residual disease, prevent recurrences and inhibit incipient metastases after conventional tumour management[8].

Tumour prevention by specific immunity

Recent studies have led to the discovery of gene mutations that predispose to cancer. Thus, it might be possible to detect susceptible individuals with a defined genetic prognosis[9]. Identification of the gene at risk and its mutated or amplified products would provide a heaven-sent opportunity to vaccinate susceptible subjects against their foreseeable cancer. The molecular characterization of altered gene products predicted to become tumour antigens will be the first step towards engineering effective vaccines for this purpose.

Identification of human tumour antigens has revealed that a few of them are expressed by distinct tumours[10]. If the most common antigens are found to number ~50, vaccination of healthy individuals against tumours will become more feasible. Immunologic intervention has been clearly shown to prevent the onset of virus-related tumours, such as Marek's disease of poultry[11], and human hepatocellular carcinoma[12], where vaccination prevents cancer by eliminating the main risk factor.

The question whether immunologic approaches can be successful once a cell population has been subjected to the initial carcinogenic hit, has rarely been examined. However, vaccination could plausibly induce a strong immune response against ignored or fully tolerated antigens associated with the most common tumours that arise within a population. Owing to the polymorphism of the glycoproteins of the major histocompatibility complex (MHC), different vaccines would need to be prepared to fit the polymorphic peptide-binding clefts. It is likely that certain tumour antigens will have a restricted usage and a few individuals will not be easily vaccinated.

These are practical and perhaps solvable problems. The real issue is whether inducing an efficient immune response will offer protection against spontaneous tumours. The central tenet of tumour immunology is that recognition of tumour antigens is followed by the establishment of a long-lasting immune memory and the specific killing of tumour cells. This notion is supported by experimental data from many transplantable tumours. The use of appropriate vaccines has shown that even spontaneous tumours, originally thought to be nonimmunogenic[13], can induce protection against subsequent challenge[14]. Nevertheless, very few data are available on spontaneous tumours, which display a longer and more complex natural relationship with their host than transplantable forms.

Mice transgenic for oncogenes might act as models to explore the defensive role of the immune system in tumourigenesis. For example, mice transgenic for the rat *neu* oncogene are protected from tumour development when vaccinated with the DNA encoding the extracellular domain of the *neu* p185 product[15] and soluble p185 protein[16], suggesting that they hamper the onset of tumours. The challenge is to pass from a proof of principle to an effective human vaccine.

Antigen-loss variants are unlikely to emerge as tumour escape mechanisms when the target molecule is directly linked to neoplastic transformation and progression, as in the case of p185 (P-L. Lollini *et al.*, unpublished) and other oncogene products. A more probable escape route is offered by the defects in antigen processing and MHC class I downregulation detected in murine[17] and human carcinomas[18].

Nonspecific immunity strikes back

Blockade of tumour growth through nonspecific stimulation of the immune system is a notion as old as it is naive. The molecular definition of many nonspecific reaction mechanisms, however, has corrected many prejudices. Straightforward comparison shows that nonspecific mechanisms possess a much greater curative potential than those elicited by specific immunity. Only a minority of mice challenged with an aggressive mammary carcinoma (TSA) were cured by repeated immunizations with cytokine-gene-engineered TSA cells[3,5] or TSA peptide-pulsed dendritic cells[1] begun immediately after the challenge, and almost none were cured when immunizations began on day 7 – however, the great majority of these 7-day-old tumours were cured by repeated injection of low doses of interleukin 12 (IL-12)[5,8]. Tumour destruction results from three major mechanisms: (1) destruction of tumour vessels by polymorphonuclear leukocytes; (2) indirect inhibition of angiogenesis by secondary interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and third-level chemokines; and (3) activation of leukocyte subsets capable of producing proinflammatory cytokines, cytotoxic T lymphocytes and antitumour antibodies[8].

Surprisingly, similar IL-12-triggered mechanisms inhibit both chemical[19] and *neu*-dependent[20] carcinogenesis. When BALB/c mice were injected subcutaneously with 3-methylcholanthrene, 100 ng IL-12 administered systemically 5 days/week for 18 weeks (3 weeks on, 1 week off) delayed tumour appearance and reduced tumour incidence. Secondary IFN-γ, IL-10 and TNF-α were induced throughout the treatment. High production of IFN-γ by $CD8^+$ T cells, and a T helper 2 (Th2) to Th1 or Th0 shift in the cytokine secretion profile of $CD4^+$ T cells were also seen in the treated mice[19].

The mammary glands of female BALB/c mice carrying the activated HER-2/*neu* oncogene and adult female FVB mice carrying the HER-2/*neu* protooncogene progress through atypical hyperplasia to *in situ* and

invasive lobular carcinoma. This progression begins in BALB/c-*neu* mice when they are weaning and in FVB-*neu* mice when they are adults. Systemic treatment of mice with preneoplastic lesions with IL-12 5 days/week (1 week on, 3 weeks off; first course 50 ng IL-12/day, the remainder 100 ng/day) markedly delayed tumour onset and reduced tumour multiplicity. Analogous results were obtained in immunocompetent and permanently $CD8^+$ T-cell-depleted mice. In both transgenic lines, tumour inhibition was associated with mammary infiltration by reactive cells, production of cytokines and inducible nitric oxide synthase (iNOS), reduction in microvessel number and a high degree of haemorrhagic necrosis[20].

These experiments suggest that stimulation of nonspecific immunity can prevent tumour formation – an unexpected and indeed provocative deduction. The resemblance of methylcholanthrene and HER-2/*neu* carcinogenesis models to human tumours indicates that nontoxic IL-12 regimens might constitute a significant prophylactic strategy. Generalization of these findings to other tumours and cytokines could allow stimulation of nonspecific immunity to be used to protect individuals with a genetic risk of cancer (Fig. 1a) and those with preneoplastic lesions (Fig. 1b,c) as a 'soft' immunologic alternative to controversial and distasteful preventive measures[9].

Apparently, IL-12 inhibits cancerogenesis by slowing down the transition from preneoplastic to overt tumours, halting angiogenesis and activating tumour-associated leukocytes through the induction of several secondary cytokines and mediators[19,20]. Nonspecific immunity can probably never lead to tumour eradication, particularly because some of its effector mechanisms, including its anti-angiogenic effects, are cytostatic rather than cytotoxic. They certainly appear to delay the appearance of tumours and, in some human situations, this could almost be regarded as equivalent to a cure[21]. Many chemopreventive agents are currently under investigation, including several new selective oestrogen receptor modulators[22]. In the future, combined chemical and immunologic preventive management might significantly decrease the incidence of clinically evident tumours in individuals at risk.

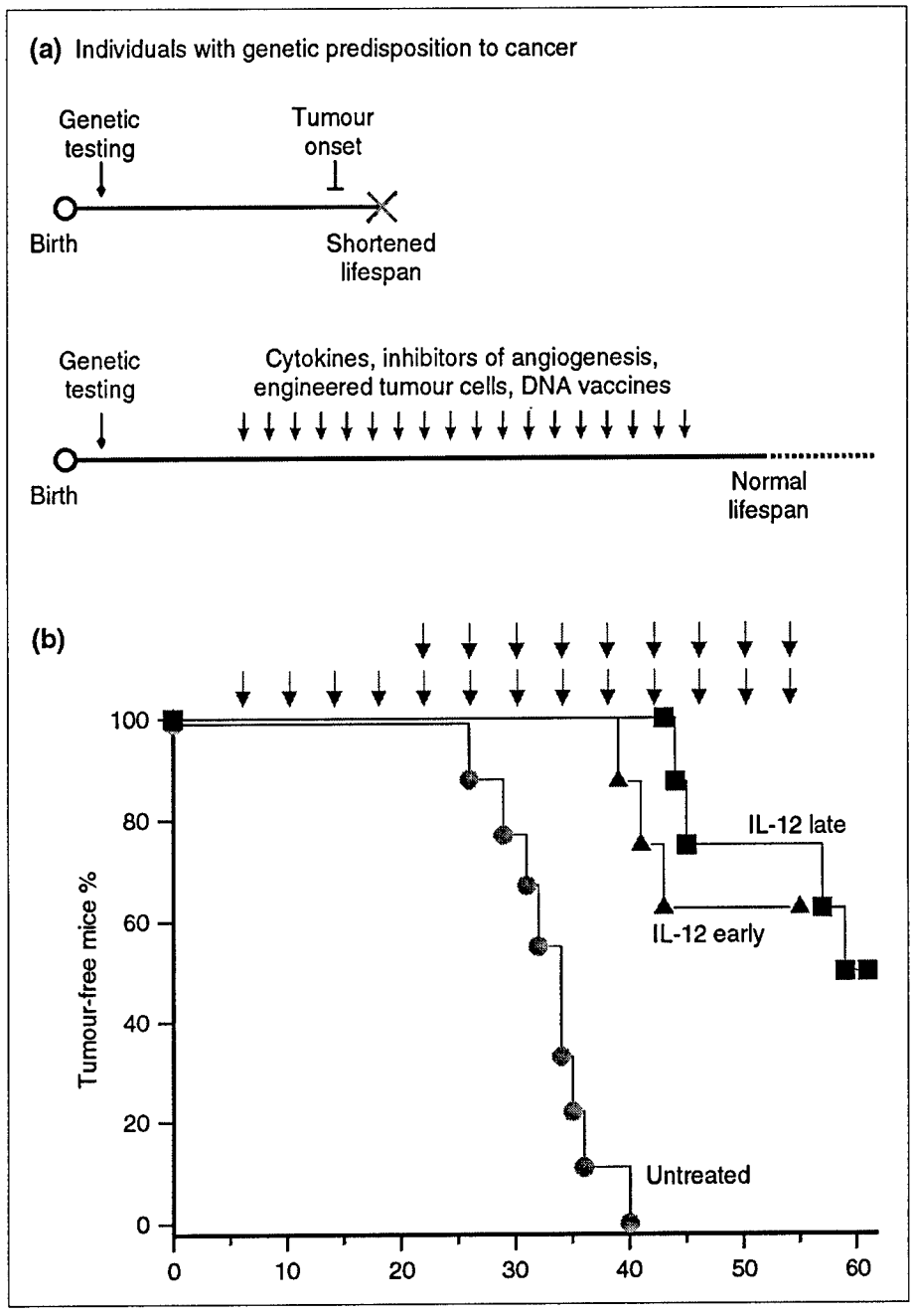

Fig. 1. *(a) The tumour prevention modalities currently available to individuals inheriting a genetic predisposition to cancer are distasteful and controversial[9,23]. An alternative is to interrupt tumour progression by manoeuvres that stimulate nonspecific host immune responses and inhibit tumour angiogenesis[19,20]. The graph (b) depicts an experimental proof of this concept. Transgenic FVB mice expressing the HER-2/neu protooncogene in the mammary gland invariably develop malignant carcinomas with a long latency period. A chronic treatment with recombinant interleukin 12 (rIL-12; each arrow represents one week of IL-12 treatment) significantly reduced tumour incidence (for further details, see text and Ref. 20). Tumour progression was prevented by IL-12 treatment started both in young mice (IL-12 early) and in adult mice (IL-12 late). In this combination of cancer predisposition and immunoprophylactic approach, a lifetime treatment was not necessary, thus sparing potentially harmful side-effects during young age.*

Conclusions

Molecular data on tumour antigens and elucidation of the protective role of the immune system in tumourigenesis might provide new strategies in oncology. The immunotherapeutic path trodden so far has not had much experimental backing and may be too hard to follow; its therapeutic success has been undeniably marginal. Nevertheless, it was fully

justified by the seriousness of the problem and led to many scientific discoveries. Development of antitumour vaccines and investigation of the defensive role of nonspecific immunity in tumourigenesis will not be easy. However, it might be rewarded by the creation of effective tumour prevention strategies.

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Analysis of Mammary Carcinoma Onset and Progression in HER-2/*neu* Oncogene Transgenic Mice Reveals a Lobular Origin

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SUMMARY: Morphologic examinations of mammary neoplasias arising in BALB/c (H-2^d) mice carrying the activated rat HER-2/*neu* oncogene (BALB-NeuT), and in FVB (H-2^q) mice bearing the wild-type proto-oncogene (FVB-NeuN), indicate that both conditions result in a very human-like lobular carcinoma of alveolar type, whose histotype is the result of the preferential expression of HER-2/*neu* products in the epithelium of lobular ducts and lobules. Detailed analysis of tumor progression indicates that transition from lobular hyperplasia to overt carcinoma is associated with a high epithelial proliferation rate, as assessed by anti-proliferating cell nuclear antigen immunostaining, and coincides with the activation and maximal extension of tumor angiogenic process as assessed by microvessel count (anti-CD31), anti-β_3 integrin, and anti-laminin immunostaining. Neovascularization is accompanied by vascular endothelial cell growth factor and basic fibroblast growth factor production by hyperplastic epithelial cells. By contrast with the BALB-NeuT tumors, E-cadherin expression is almost nonexistent in those arising in FVB-NeuN mice and this may explain their high metastatic potential. Despite their different kinetics, however, the lung metastases observed in both strains are histologically similar and resemble the primary tumor. Both strains can thus be proposed as models for "in vivo" investigation of the origin and progression of the alveolar type of lobular mammary carcinoma and the testing of new therapeutic approaches. (*Lab Invest 1999, 79:1261–1269*).

Breast cancer is the most frequent malignancy of woman worldwide (Parkin et al, 1999). Rodent models have been particularly useful in illustrating its pathogenesis and evaluating its response to therapy (Anderson, 1992). These models, however, do not reflect the complex variety of human mammary cancer, because they are almost exclusively virus or chemically induced ductal adenocarcinomas (Russo and Russo, 1996). Generation of mouse strains transgenic for the HER-2/*neu* oncogene offers the opportunity to investigate a spontaneously arising mammary carcinoma and evaluate the "in vivo" role of HER-2/*neu* in cancerogenesis and progression (Bouchard et al, 1989; Guy et al, 1992, 1996; Lucchini et al, 1992; Muller et al, 1988; Suda et al, 1990).

The HER-2/*neu* oncogene is involved in human mammary cancerogenesis. Its amplification and overexpression, in fact, have been observed in a large percentage of primary human breast cancers and seem to be inversely correlated with survival (Di Giovanna et al, 1996; King et al, 1985; Slamon et al, 1987, 1989), though the significance of this correlation varies widely from one study to another.

Previous genetic, biochemical, and morphologic studies of HER-2/*neu* in mouse mammary carcinogenesis have provided a schematic representation of its contribution to tumor progression in both mice and humans (Di Giovanna et al, 1998). However closer histologic and pathologic investigation of HER-2/*neu*–associated tumor onset and progression is needed to determine the extent to which the mouse and human forms converge and diverge.

In this article we report that BALB/c transgenic female mice carrying the activated rat HER-2/*neu* oncogene (Boggio et al, 1998; Muller et al, 1988) quickly develop mammary tumors pathologically similar to those developed more slowly by transgenic FVB female mice carrying the wild-type proto-oncogene and overexpressing its product (Guy et al, 1992). Both

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Appendix 2

tumors are similar to the alveolar-type human lobular mammary carcinoma.

Inclusion of a lobular type in the histologic classification of rodent mammary tumors (Russo and Russo, 1996) makes it more detailed and fully comparable to that of human forms (Rosai, 1996). Herein we propose these two strains of HER-2/*neu* transgenic mice as a model for investigation of the mechanisms underlying the origin and progression of lobular breast cancer.

Results

Histologic Examination of HER-2/neu Transgenic Mice Mammary Tissue

Female BALB/c (H-2^d) mice carrying the activated rat HER-2/*neu* oncogene (BALB-NeuT) showed no palpable lesions of the mammary gland until 15 weeks of age. They then began to develop multiple mammary tumors that progressively involved all 10 glands by the 33rd week. No appreciable differences in tumor natural history were observed in the HER-2/*neu* transgenic CD1 mice mated with BALB/c to obtain BALB-NeuT mice. Female FVB (H-2^q) mice carrying the HER-2/*neu* proto-oncogene with HER-2/*neu* product overexpression (FVB–NeuN) developed mammary carcinomas with a longer latency (38th to 49th week) and a lower multiplicity (mean of 2.6 tumors/mice).

Histologic examination of BALB-NeuT mammary tissue showed that widespread atypical hyperplasia of small lobular ducts and lobules was already evident at 3 weeks (Fig. 1a) and characterized by proliferation of a relatively uniform population of round epithelial cells assuming a stratified appearance with no formation of epithelial bridges.

Starting at the 11th week, the ductules and acini within the lobules were distended by the solid, occlusive growth of this epithelial cell population (Fig. 1b). The myoepithelial cell layer was scarcely represented or absent around the neoplastic lobular structures. The ductular and acinar outlines remained distinct and separate from one another, with persistence of intervening stroma. These features were distinctive of lobular carcinoma "in situ." At nearly the 20th week, alveolar groups of neoplastic cells with no myoepithelial lining infiltrated the surrounding adipose tissue (Fig. 1c). The linear, "Indian-file" arrangement of tumor cells and their circumferential growth around ducts and lobules ("targetoid growth") (Rosen and Oberman, 1993) were not observed. Histologic examination performed in transgenic CD1 mice revealed the development of mammary lobular carcinoma with morphologic features similar to those found in BALB-NeuT mice (data not shown). Thus the genetic background of BALB/c did not alter the carcinogenesis and the tumor phenotype in transgenic CD1 mice.

Histologic examination of FVB-NeuN mammary tissue revealed normal ductular and lobular structures until 35 to 37 weeks, after which foci of epithelial hyperplasia evolving to lobular carcinoma "in situ" and then to invasive lobular carcinoma of the alveolar type were found. The histologic features of this carcinoma were similar to those observed in BALB-NeuT mice, though the proliferating cell population displayed minor variations in size and in cytoplasm staining. Lung metastases recovered from both strains were histologically similar to the primary tumor.

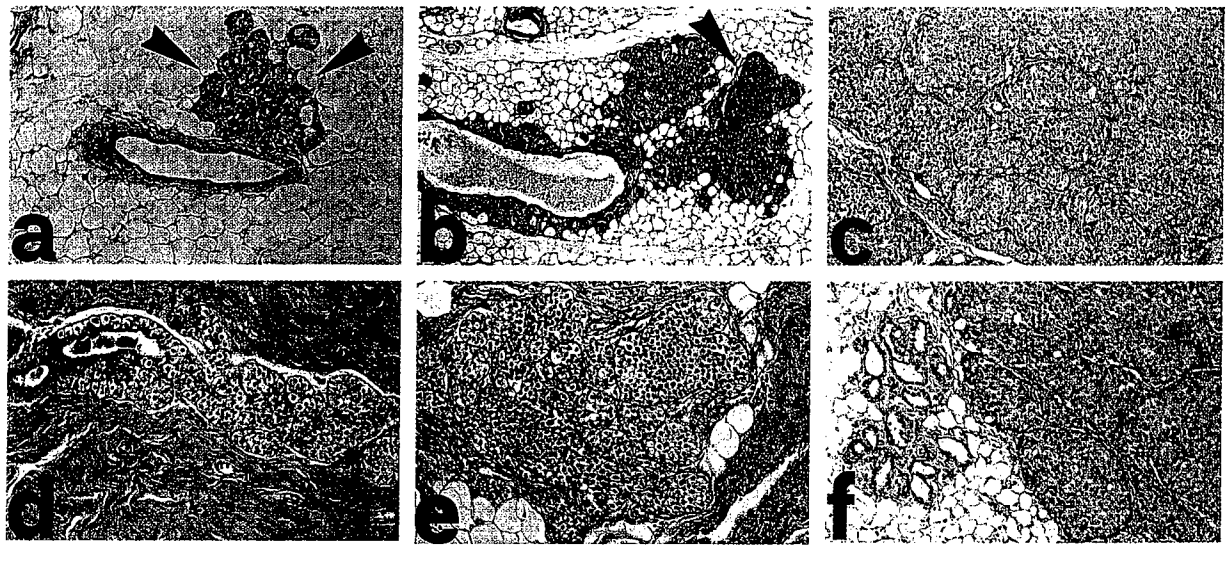

Figure 1.

Histologic features of lobular carcinoma development in rat HER-2/*neu* transgenic mice (a–d). In 5-week-old BALB-NeuT mice, small lobular ducts and lobules (*arrowheads*) are almost completely occupied by round epithelial cells assuming the stratified appearance of hyperplasia (a). In 13-week-old BALB-NeuT mice, the neoplastic epithelial cell proliferation assumed the solid occlusive intralobular growth typical of the lobular carcinoma in situ (*arrowhead*) (b). Typical pattern of lobular carcinoma with alveolar arrangement in BALB-NeuT (c) and in FVB-NeuN (f) mice. The histologic features of human mammary lobular atypical hyperplasia (d) and carcinoma (e) are quite similar to those arising in HER-2/*neu* transgenic mice. In d, the normal epithelium of a small lobular duct and the contiguous lobular structures are almost completely replaced by a solid occlusive proliferation of a relatively uniform population of round cells with a pale cytoplasm. (Hematoxylin and eosin staining; original magnification of a, c, d, e, f ×200; b ×100.)

Ultrastructural Examination

Ultrastructural examination of BALB-NeuT and FVB-NeuN tumors showed that most cells had a pale-staining, organelle-poor cytoplasm and a large oval nucleus with evenly distributed chromatin (Fig. 2, a, b, and d). Occasionally in BALB-NeuT and frequently in FVB-NeuN mice, tumor cells had darker cytoplasms and more irregular nuclei. The cells were linked by poorly developed junctions. In lobular carcinoma "in situ," a thin and discontinuous layer of myoepithelial cells and a basal lamina surrounded almost all tumor-containing ductules and alveoli. Invasive lesions were accompanied by a loss of myoepithelial cells and basal lamina. The histologic and ultrastructural features of these carcinomas were identical to those of human lobular carcinoma (Fig. 1, d and e, and Fig. 2c).

Immunohistochemistry

Immunohistochemistry with anti-*neu* antibody showed that the epithelial cells of extralobular ducts were mainly negative, whereas those of nonneoplastic lobular ducts and lobules and of neoplastic lobular lesions displayed a strong cell membrane staining (Fig. 3, a and b).

Proliferating cell nuclear antigen (PCNA) was expressed by the majority (65.2% ± 13.1%) of epithelial cells in hyperplastic ductular and lobular structures (Table 1 and Fig. 3c), whereas only 17.8% ± 3.1% of cells of extralobular ducts were positive. Its expression in lobular carcinomas (Fig. 3d) was mainly detected in the peripheral cell layer of neoplastic lobules (24.8% ± 7.3% of epithelial cells).

Intercellular E-cadherin expression was found in normal and hyperplastic mammary glands from both BALB and FVB transgenic mice. It was still detectable in BALB-NeuT, but not in FVB-NeuN lobular carcinomas (Fig. 3, e and f).

Studies were also performed in BALB transgenic mice to investigate angiogenesis during tumorigenesis. Before (2nd week) and during hyperplasia (5th week), and when lobular carcinoma in situ (15th week)

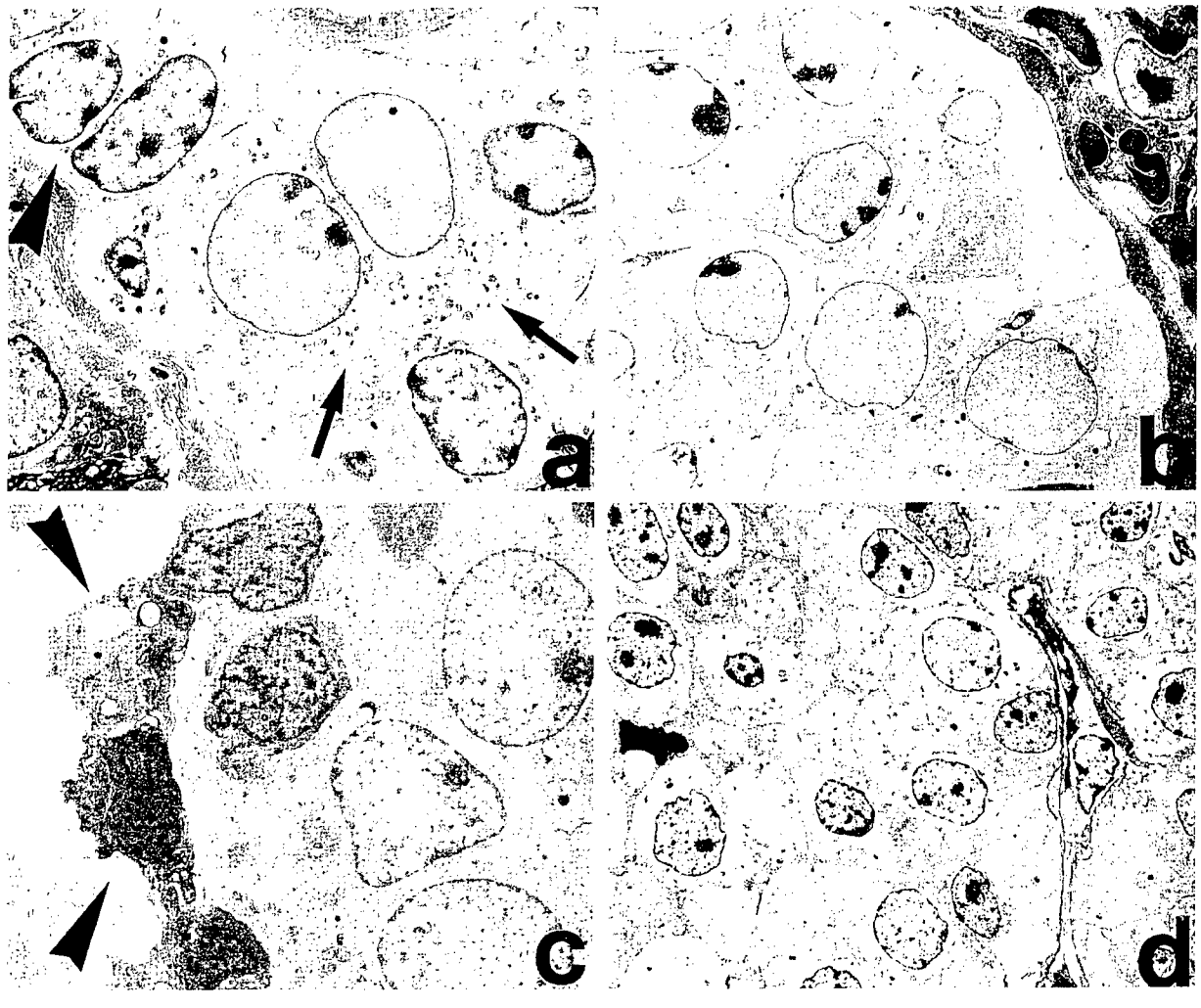

Figure 2.
Ultrastructural features of hyperplastic ductular structure in BALB-NeuT mice in which proliferating neoplastic cells (*arrows*) replace the normal epithelium (*arrowhead*) (a). Lobular carcinoma in BALB-NeuT (b) and FVB-NeuN mice (d) constituted of round to polygonal cells with an organelle-poor cytoplasm and a large round or oval nucleus (b). Human lobular carcinoma in situ (c) with neoplastic cells quite similar to those of mouse lobular carcinoma. Myoepithelial cells (*arrowheads*) lining the neoplastic lobular structure are tightly close to the basal lamina. (a, c original magnification, ×2,750; b ×1,900; d ×1,450.)

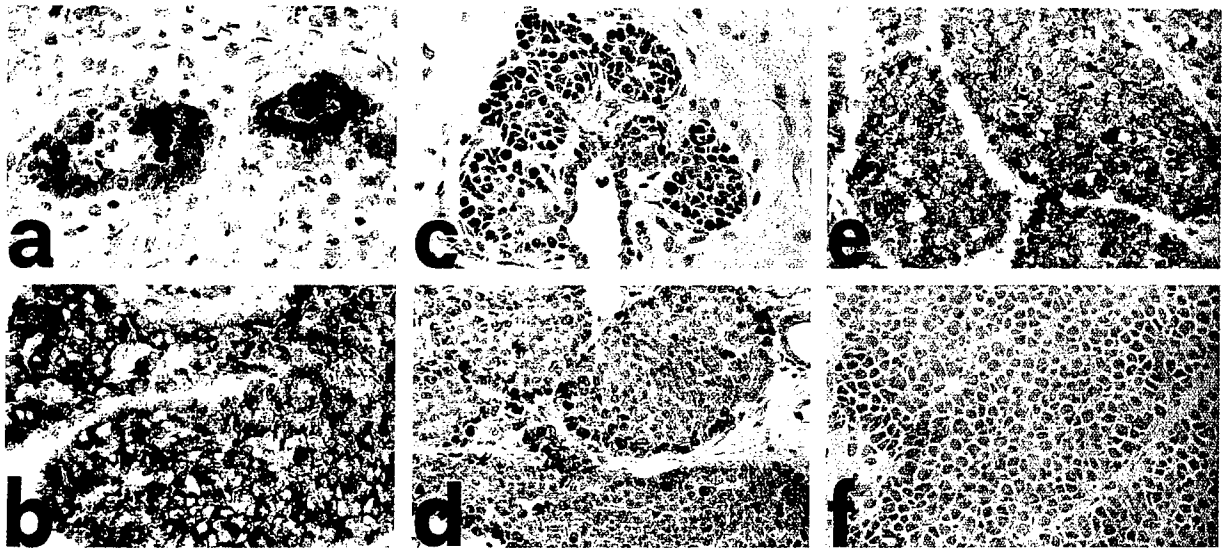

Figure 3.

Immunohistochemistry performed with anti-*neu* antibody revealed a strong positivity of epithelial cells in hyperplastic (a) and neoplastic (b) lobular lesions. Proliferating cell nuclear antigen is expressed by the majority of epithelial cells in hyperplastic ductular and lobular structures (c), whereas in lobular carcinoma it is mainly detected in the peripheral cell layer of neoplastic lobules (d). Intercellular E-cadherin expression is evident in lobular carcinoma of BALB-NeuT (e), but not in that of FVB-NeuN (f). (a–f original magnification, ×630.)

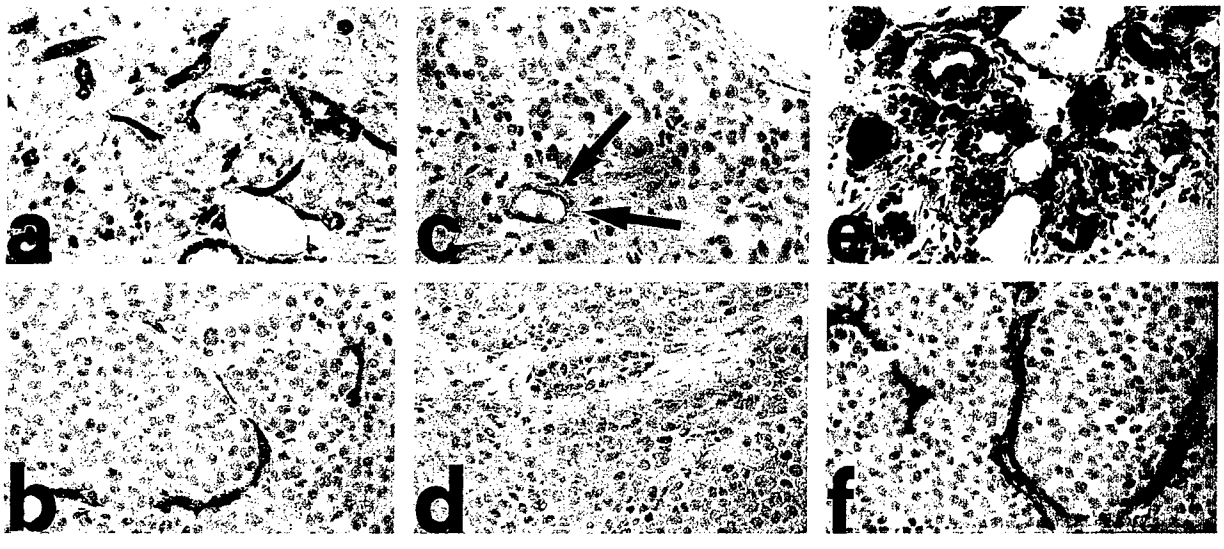

Figure 4.

Cryostat sections tested with anti-endothelial cells antibody (anti-CD31) showing that hyperplastic foci (a) are much more vascularized than carcinomatous mammary tissue (b). Some capillaries (*arrows*) present in hyperplasia (c) express the β_3 subunit of the $\alpha_v\beta_3$ receptor, which is almost absent in lobular carcinoma (d). Basic fibroblast growth factor (bFGF) is clearly expressed by epithelial cells during hyperplasia (e), whereas in lobular carcinoma, a marked bFGF staining is evident in the extracellular matrix bordering the neoplastic lobular structure (f). (a–f original magnification, ×630.)

and invasive alveolar lobular carcinoma (28th week) developed, tissue specimens were tested with anti-endothelial cells (CD31), anti-basement membrane components (anti-laminin and anti-collagen type IV), and anti-β_3 chain antibody, which recognizes the adhesion receptor $\alpha_v\beta_3$ selectively expressed on growing vessels.

Microvessel counts indicated that hyperplastic foci were much more vascularized than nonhyperplastic or carcinomatous tissue (Table 1 and Fig. 4, a and b). Several capillary sprouts in hyperplastic foci expressed the β_3 chain of the $\alpha_v\beta_3$ receptor (Fig. 4c) which was absent in normal mammary tissue. The capillary basement membrane component laminin showed a fibrillar distribution (data not shown) instead of the linear pattern found in quiescent mature vessels. A scanty presence of β_3 and a well-defined and continuous pattern of basal lamina components were observed in lobular carcinomas, in which both extracellular matrix molecules laminin and collagen type IV were more represented than in hyperplasia.

Immunohistochemical staining for angiogenic factors demonstrated that vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF), absent or scarcely present in normal mammary tissue, were clearly expressed by epithelial

Table 1. Microvessel Count, Rate of Proliferating Cells, and Expression of β_3 Integrin, Angiogenic Factors, and Extracellular Matrix Components in Hyperplastic and Carcinomatous Tissues of BALB-NeuT Mice

	BALB-NeuT mice	
	Hyperplasia (5 wk of age)	Lobular carcinoma (15 wk of age)
Microvessel count	27.0 ± 3.2[a]	15.9 ± 2.1*
PCNA immunoreactivity rate	65.2% ± 13.1%	24.8% ± 7.3%*
β_3 Integrin	++[b]	+/−
bFGF	++	+
VEGF	+	+/−
Laminin	+	++
Collagen type IV	+	++

[a] Microvessel counts were performed on cryostat sections tested with anti-endothelial (CD31) Ab and the rate of proliferating cells (PCNA immunoreactivity) was evaluated on formalin-fixed, paraffin-embedded tissue sections with anti-PCNA Ab as described in Materials and Methods. At least 10 fields were counted per sample. Values are expressed as mean ± SD of five 5-wk-old and five 15-wk-old mice.

[b] The expression of β_3 integrin, angiogenic factors, and extracellular matrix components was defined as absent (−), or scarcely (+/−), moderately (+), or frequently (++) present on cryostat sections tested with the corresponding antibody.

* Value significantly different ($p > 0.001$) than that of hyperplasia.

PCNA, proliferating cell nuclear antigen; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor.

cells during hyperplasia (Fig. 4e and Table 1). It also showed that their corresponding proteins were mainly confined in the basal neoplastic epithelial cell layer. A marked bFGF staining was evident in laminin and collagen type IV rich extracellular matrix bordering the neoplastic lobular structures (Fig. 4f).

Detection of Estrogen and Progesterone Receptors

A low level of estrogen receptors (ER) (8.1 fmole; range, 7.4 to 9.3) and progesterone receptors (PR) (16.2 fmole/mg cytosol protein; range, 14.8 to 17.7) was found in BALB-NeuT carcinomatous tissue. The K_d values for both receptors ranged from 0.1 to 0.2 nM.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Expression of bFGF and VEGF was also demonstrated by reverse transcriptase-polymerase chain reaction in BALB-NeuT carcinomatous tissue at the mRNA level (Fig 5).

Discussion

Expression of the activated rat HER-2/*neu* oncogene in BALB-NeuT mice results in the rapid and synchronous development of multifocal mammary tumors, whereas FVB-NeuN mice carrying the wild-type HER-2/*neu* oncogene develop mammary carcinomas asynchronously with a longer latency and lower multiplicity (Boggio et al, 1998; Muller et al, 1988). Despite these kinetic differences, histologic and ultrastructural examination of neoplasias indicates that expression of activated or overexpression of the wild type of rat HER-2/*neu* oncogene leads to the development of lobular carcinomas. This identity is also in agreement with the finding that the HER-2/*neu* proto-oncogene is frequently activationally mutated in FVB-NeuN transgenic mice (Siegel et al, 1994).

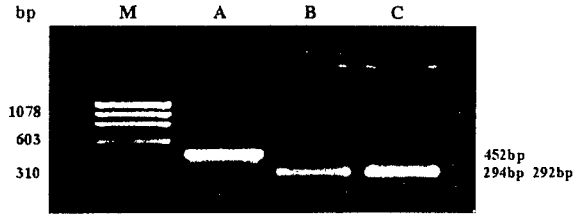

Figure 5.
Expression of vascular endothelial cell growth factor (VEGF) (294 bp; lane B) bFGF (292 bp; lane C) in mRNA extracted from BALB-NeuT lobular carcinoma. Total RNA was isolated from mammary tissue obtained from a 25-week-old mouse. The 292-bp bFGF band is wider than that of VEGF (294 b). G3DPH message (452 base pairs [bp]; lane A) served as control. The marker (lane M) is *Hae*III digest of Phi ×174.

Diagnosis of these lobular carcinomas is based on replacement of the normal epithelium of acini and intralobular ductules by neoplastic cells. This conclusion is derived from our weekly histologic and immunohistochemical evaluation of HER-2/*neu* mammary tissue, which initially displays hyperplasia spreading all over the lobular structures, followed by an "in situ" and then an invasive lobular carcinoma. Furthermore epithelial proliferation within the lobular structures is characterized by solid, occlusive proliferation of a relatively uniform population of loosely cohesive, and mainly small, round cells with sparse cytoplasm distending the acini. At the ultrastructural level, the neoplastic cells display an organelle poor cytoplasm with oval, pale nuclei and inconspicuous nucleoli. These histologic and ultrastructural findings are identical to those observed in human mammary lobular carcinoma (Eusebi et al, 1977; Murad, 1971; Nesland et al, 1995; Rosen and Oberman, 1993). The acinar outlines remain distinct and separate from one another with persistence of intervening delicate stroma. It is important to note that the carcinoma in both strains is multicentric, as often reported in human lobular carcinoma.

Pathologists probably have not defined these tumors as lobular (Bouchard et al, 1989; Guy et al, 1996; Munn et al, 1995) because they mainly focused on the cytologic aspects of transformed epithelial cells which were identified as "intermediate cells" (Cardiff et al, 1991), ie, clear or basal cells supposed to originate from a metaplastic alteration occurring in mammary epithelial or myoepithelial cells. Furthermore the tendency of tumor cells to grow in a solid, loosely cohesive manner might be attributed to a relative, ultrastructurally observed, preservation of cell-to-cell junctions, that probably prevent the establishment of the "Indian file" arrangement and the "targetoid" growth pattern frequently found in invasive human lobular carcinoma (Rosen and Oberman, 1993). Cer-

tainly, the absence of these morphologic aspects does not make the diagnosis of lobular carcinoma easier. Moreover the occasional presence of central necrosis inside the neoplastic lobules mimics a characteristic feature of ductal mammary carcinoma (Rosai, 1996). A further reason for the lack of definite histologic characterization could be the frequent association in humans of HER-2/*neu* overexpression with ductal carcinoma (25% to 40%), whereas in lobular carcinoma this association is rarely found (1% to 13%) (Porter et al, 1991; Querzoli et al, 1998; Zschiesche et al, 1997).

In an attempt to better define the phenotypic profile of the transformed epithelial cells in mammary carcinoma of BALB-NeuT mice, we found a low level expression of ER and PR. Early studies in humans suggested that invasive lobular carcinoma was exceptionally ER-rich, but this has not been substantiated in larger groups (Lesser et al, 1981). High levels of ER and PR were found in 12 patients with the alveolar variant of invasive lobular carcinoma, though values ranged from more than 300 to 1495 fmole/mg of cytosol protein (Du Toit et al, 1989; Shousha et al, 1986).

The E-cadherin molecule is expressed at the surface of epithelial cells and plays a crucial role in epithelial organization and adhesion (Takeichi, 1991). Its expression frequently is reduced in human mammary lobular carcinomas (Vos et al, 1997), mainly in those with a more pronounced metastatic potential (Berx et al, 1995; Siitonen et al, 1996). Lobular carcinoma from BALB-NeuT mice strongly expressed E-cadherin, whereas it was almost undetectable in FVB-NeuN mammary tumors. This latter feature could explain why Guy et al (1992) found that the overexpressed rather than the activated HER-2/*neu* gene enhances the metastatic potential of the mammary tumor cell.

The major functional units of the mouse mammary gland are termed lobulo-alveolar units or terminal end buds, which are regarded as equivalent to the terminal ductal lobular units of the female human breast (Cardiff, 1998; Russo and Russo, 1996).

There is evidence that spontaneous and chemically induced ductal tumors develop in lobulo-alveolar/terminal end buds units (Cardiff, 1998; Russo and Russo, 1996). Because these units contain the proliferative stem cell populations most sensitive to the effects of somatic cell mutation, they seem to be the site of origin for most mammary cancers, including those of lobular type. It has been hypothesized that human lobular carcinoma arises from a more complex and differentiated lobular structure (lobule type 2) that evolves from terminal ductal lobular units (Russo and Russo, 1996).

In BALB and FVB transgenic mice, the genetic alteration may lead to proliferation of epithelial cells in the already well-differentiated lobular structures, similar to lobule type 2, which contains almost all cells expressing HER-2/*neu* product. HER-2/*neu*–triggered epithelial cell proliferation is evidenced by the widely distributed expression of PCNA in these more differentiated lobular structures. Conversely, neoplastic cell proliferation starting in the lobulo-alveolar/terminal end buds units may give rise to the more complex lobular arrangement. This pathogenic pathway is probably based on the multifocal and widely distributed presence of HER-2/*neu*–expressing transformed cells.

Previous studies in transgenic mice reported that tumorigenesis proceeds through two stages (Folkman et al, 1989; Parangi et al, 1996). The first involves oncogene product expression which leads to hyperplasia, the second consists of angiogenesis induction. Our findings provide a further illustration of this pattern. In our model there seems to be a close connection between hyperplasia, characterized by an increase in epithelial cell proliferation, and the activation of angiogenesis. We have morphologic evidence that in hyperplastic foci, angiogenesis begins before overt tumor formation. In hyperplasia, we observed an increased number of microvessels in the stroma surrounding the hyperplastic small lobular ducts and lobules. Several microvessels expressed the b_3 subunit of $\alpha_v\beta_3$ integrin, which has been reported to promote endothelial cell migration, angiogenesis, and protection from apoptosis (Brooks et al, 1994a, 1994b; Shattil, 1995). Its expression identifies new vessel sprouts and is a real indicator of neovascularization (Brooks et al, 1994a).

Neovascularization is probably activated by bFGF- and VEGF-producing hyperplastic epithelial cells. These angiogenic factors were also expressed in lobular carcinoma confined to the basal neoplastic epithelial cell layer close to the intervening stroma. It has been reported that bFGF molecules stored and immobilized in the extracellular matrix are normally inactive because of their strong adherence to heparin sulfate proteoglycans (Czubayko et al, 1997; Rak and Kerbel, 1997). During tumor progression, therefore, the extracellular matrix could sequester bFGF and impede its angiogenic effects. The mean number of microvessels per microscopic field, in fact, was appreciably reduced in lobular carcinoma compared with the preceding hyperplasia, in which the extracellular matrix constituents (laminin and collagen type IV) were less represented.

Discussions on spontaneous or chemically induced mammary tumors have never reported a lobular type of carcinoma in rodents (Munn et al, 1995; Russo and Russo, 1996), whereas the ductal type has been widely and perhaps solely described.

Apart from two casual observations (Kordon et al, 1993; Pazos et al, 1998), the finding of a lobular carcinoma in the two strains of transgenic mice studied in this work, adds a new histotype to the current histologic classification of rodent mammary epithelial neoplasms. Diagnosis in rodents of a lobular carcinoma of the alveolar type resembling that occurring in women, and our finding of its peculiar pattern of neoangiogenesis may be considered a substantial clue for anticancer research and supply an appropriate tool for the testing of new therapeutic strategies.

Materials and Methods

Mice

A transgenic CD1 random-bred breeder male mouse (no. 1330) carrying the mutated rat HER-2/*neu* oncogene driven by the mouse mammary tumor virus promoter (Tg-NeuT, provided by Dr. L. Clerici, Euratom, Ispra, Italy) (Lucchini et al, 1992) was mated with BALB/c females (H-2^d; Charles River, Calco, Italy). The progeny was screened for the transgene by PCR. Transgene-carrying males were backcrossed with BALB/c females for more than 12 generations and HER-2/*neu* BALB/c mice (BALB-NeuT) were used in these experiments. Parental FVB-NeuN N#202 transgenic mice carrying the rat HER-2/*neu* proto-oncogene driven by the mouse mammary tumor virus promoter on the H-2^q FVB inbred background were provided by Dr. W. J. Muller (McMaster University, Hamilton, Ontario, Canada) and bred in our animal facilities. Females of both lines show a mouse mammary tumor virus-driven overexpression of the transgene in the mammary gland and a definite tumor growth involving its epithelium (Guy et al, 1992, 1996; Lucchini et al, 1992). Individually tagged virgin females were used in this study. Starting at the age of 5 weeks, their mammary glands were inspected once a week, and masses were measured with calipers in the two perpendicular diameters (Guy et al, 1992). Progressively growing masses >3-mm mean diameter were regarded as tumors.

Histologic and Ultrastructural Analysis

Groups of two or three BALB-NeuT mice were killed at Week 2 and 3 and then every other week until Week 33 of age; similar groups of FVB-NeuN were killed every 4 weeks from 5 to 61 weeks of age. For histologic evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For electron microscopy, specimens were fixed in cacodylate-buffered 2.5% glutaraldehyde, postfixed in osmium tetroxide, and then embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate-lead citrate.

Immunohistochemistry

For immunohistochemistry, formalin-fixed, paraffin-embedded or acetone-fixed cryostat sections were incubated for 30 minutes with anti-endothelial cells (mEC-13.324) (Vecchi et al, 1994), anti-CD61 (integrin β_3 chain) (PharMingen, San Diego, California), anti-collagen type IV (Chemicon, Temecula, California), anti-laminin (Becton Dickinson, Bedford, Massachusetts), anti-VEGF, anti-bFGF and anti-Neu (C-18) (Santa Cruz Biotechnology, Inc., Santa Cruz, California), anti-PCNA (Ylem, Rome, Italy), and anti-uvomorulin (E-cadherin) (Sigma Immunochemicals, Milan, Italy) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Ig (Vector Labs., Burlingame, California) for 30 minutes. Unbound Ig was removed by washing and the slides were incubated with avidin-biotin complex/alkaline phosphatase (Dako, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blinded manner. Two or more samples (1/tumor growth area) and 10 randomly chosen fields in each sample from mice with multiple hyperplastic foci or tumors were evaluated for each point determination. For microvessel and reactive cell counts, individual microvessels and cells were counted under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2 per field). The rate of immunoreactivity for PCNA was obtained by counting the number of positive cells/number of total cells in the ductular and lobular structures under a microscope ×600 field (×60 objective and ×10 ocular lens; 0.120 mm^2 per field).

The expression of β_3 integrin, angiogenic factors, and extracellular matrix components was defined as absent (−), scarcely (+/−), moderately (+), or frequently (++) present on cryostat sections tested with the corresponding antibodies.

Estrogen and Progesterone Receptors

ER and PR were assessed as reported by Carbone and Vecchio (1985) using the dextran-coated charcoal method, as recommended by the Italian Committee for Standardisation of Tissue Hormonal receptors assays (Piffanelli et al, 1982). The concentration and apparent equilibrium dissociation constant (K_d) of receptor sites were obtained by Scatchard analysis.

mRNA for Angiogenic Factors

Total RNA was prepared from BALB/c normal mammary tissue and from BALB-NeuT neoplastic lesions by using Ultraspec (Biotecx Laboratories, Inc., Houston, Texas). Two micrograms of RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (200 U) in 50 μl of reaction mixture with oligo dT and dNTP (GIBCO BRL, Paisley, United Kingdom). The cDNA were tested for the presence of murine glucose 3-phosphate dehydrogenase, VEGF, and bFGF sequences in PCR reactions (Gene Amp Kit; Perkin Elmer Cetus, Norwalk, Connecticut) performed in 20-μl volumes and amplified by 30 PCR cycles, by using specific primer pairs prepared by us (VEGF) or from Stratagene (La Jolla, California) (bFGF).

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Review

Cytokine gene therapy: Hopes and pitfalls

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Summary

Transduction of a cytokine gene into neoplastic cells elicits a strong inflammatory host reaction that impairs tumor growth, and a long-lasting immune memory is established following their rejection. These findings have aroused great enthusiasm and expectations. Despite their enhanced immunogenicity, however, the immune reaction provoked by repeated injections of these engineered cells can do little more than inhibit the growth of initial tumors and metastases and is only minimally effective against established forms. Better therapeutic activity is thus being sought by combining such cells with tumor cells engineered with other genes.

Key words: cytokines, gene therapy, immune response

Introduction

A few correlated approaches are currently being explored to improve cancer gene therapy [1]:

(a) Direct interference with genes that sustain cancer cell proliferation. For example, insertion of a wild-type *p53* gene in cells harboring a mutant *p53* is the subject of extensive investigation.
(b) Induction of drug sensitivity based on the introduction of 'suicide genes' coding for enzymes that convert prodrugs into toxic compounds. For example, expression of the gene coding for cytosine deaminase renders a tumor cell susceptible to 5-fluorocytosine, which is deaminated to 5-fluorouracil [2].
(c) Enhancement of drug resistance. Genes such as MDR-1 could be exploited to increase the resistance of normal hemopoietic progenitors and other normal cells to high doses of conventional cytotoxic regimens [1].
(d) Enhancement of tumor immunogenicity. Here the aim of gene transfection is to induce an antitumor immune response [3].

Many genes are involved in more than one of these approaches. For example, suicide genes of bacterial or viral origin frequently enhance the immunogenicity of engineered cancer cells because their protein products are recognized by the host's immune system [4]. Conversely, the range of activities of some cytokines used for immunological purposes extends beyond the boundary of the immune response. For instance, interferons (IFNs) and tumor necrosis factor (TNF) are direct inhibitors of tumor cell proliferation, interleukin 12 (IL-12) induces chemokines that inhibit angiogenesis, and colony stimulating factors (CSFs) promote normal hemopoiesis [3].

This review is concerned solely with gene immunotherapy, namely, the use of genes coding for cytokines to stimulate the host's immune system.

The hopes of cytokine gene therapy

The central tenet of cancer immunotherapy is that the immune system is able to destroy tumor cells and to retain a long-lasting memory of its antigenic experience if tumor antigens are efficiently recognized. At present, a molecular definition of many tumor-associated antigens has been worked out. These range from mutated oncogene products to peptides abnormally expressed by tumor cells [5]. In natural conditions, however, the immune response to those antigens is feeble or nil.

It was then realized that a marked antitumor response could be elicited by the straightforward administration of appropriate exogenous cytokines [3]. Many studies in the 1980s showed that the systemic and repeated administration of high doses of cytokines resulted in sporadic but dramatic tumor regressions. Unfortunately, this approach was associated with unacceptable general toxicity [6]. It was therefore progressively abandoned in favor of local administration of very low doses at the site of an incipient antitumor reaction [3, 7]. The experimental and clinical data obtained through this more physiological way of using cytokines provided the rationale for the gene immunotherapy that began in the early 1990s [8].

Introduction ('transduction') of genes coding for cytokines into tumor cells is a simple way of promoting the sustained local release of cytokines immediately capable of enhancing the quality and intensity of the immune response to tumors [3, 7, 8].

The hopes for cytokine gene therapy stem from studies

Table 1. Therapeutic efficacy of cytokine gene transfer against established, non-engineered rodent tumors [10–54].

Cytokine gene	Number of studies	Number of protocols	Percentage of protocols reporting therapeutic efficacy[a]			
			Null	Low	Intermediate	High
IL-1	1	1		100%		
IL-2	17	31	29%	39%	10%	23%
IL-3	2	2	50%	50%		
IL-4	6	6	33%	67%		
IL-6	6	7	43%	43%	14%	
IL-7	2	3	67%	33%		
IL-10	1	1		100%		
IL-12	6	13	23%	8%	31%	38%
M-CSF	1	1	100%			
GM-CSF	12	22	18%	36%	18%	27%
TNF-α	3	3	33%	33%	33%	
IFN-α	5	7	14%	43%	29%	14%
IFN-γ	9	11	9%	64%	18%	9%
Total	71	108	26%	40%	16%	18%

Abbreviations: GM-CSF – granulocyte macrophage colony stimulating factor; IFN – interferon; IL – interleukin; M-CSF – macrophage colony stimulating factor; TNF – tumor necrosis factor.
[a] Therapeutic efficacy was classified as: low when < 25% of mice were tumor and metastasis free; intermediate when tumor and metastasis free mice were between 25% and 50%; high when > 50% of mice were tumor and metastasis free.

Table 2. Clinical trials involving cytokine gene transfer [55–60].

Cytokine gene	Number of clinical trials	*In vitro* gene transfer: cell target				*In vivo* gene transfer
		Cancer cells	Fibroblasts	Leukocytes	Cancer cells & leukocytes	
IL-2	27 (49%)	16	4	1		6
IL-4	4 (7%)	2	1	1		
IL-6	1 (2%)	1				
IL-7	3 (5%)	1			1	1
IL-12	3 (5%)		1			2
GM-CSF	9 (16%)	6	1	1		1
TNF-α	2 (4%)	1		1		
IFN-γ	2 (4%)	2				
Combined	4 (7%)	3				1
Total	55	32 (58%)	7 (13%)	4 (7%)	1 (2%)	11 (20%)

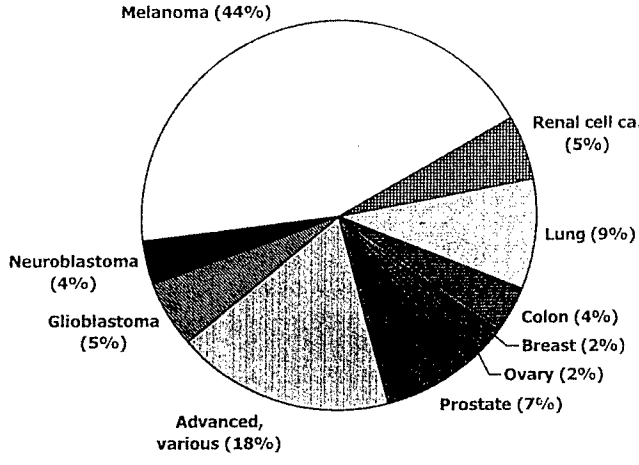

Figure 1. Tumor type.

of murine tumor cells transduced with genes coding for cytokines normally expressed by leukocytes [3, 7, 8]. In most instances, these transduced cells are no longer tumorigenic. *In vitro* they proliferate as before, but as soon as they are introduced in an immunocompetent host they attract a conspicuous leukocyte infiltrate that sweeps them away in a few days. Different cytokines elicit different proportions of granulocytes, macrophages, dendritic cells, natural killer cells, and lymphocytes. The gene therapist can therefore tailor the response required by selecting the cytokine gene appropriate for a given tumor type [9].

The reaction that destroys cytokine gene-transduced tumor cells is often followed by the establishment of a systemic, specific long-term immune memory. Animals that have rejected transduced cells are immune to a subsequent challenge with non-transduced cells from the same tumor. There are thus grounds for believing that transduced tumor cells could be used as an effective anti-cancer prophylactic vaccine [3].

The pitfalls of cytokine gene therapy: First-generation studies have shown marginal therapeutic activity

The main pitfall concealed in the results described thus far is that abrogation of the tumorigenicity of gene-transduced cells, and immunization of healthy animals against a challenge with tumor cells, are useful systems for investigating immunological mechanisms, but do not demonstrate a true therapeutic effect.

Examination of more realistic studies of the ability of cytokine genes to cure existing tumors and metastases (Table 1) shows that the truly therapeutic investigations are significantly fewer in number than those dealing with the immunological characterizations described in the previous section. The therapeutic efficacy of cytokine gene-transduced tumor cells is low. In most cases, only a minority of tumor-bearing mice were cured by the administration of cytokine gene-transduced tumor cells. Furthermore, the limited efficacy of these 'vaccines' was completely lost if they were not administered in the first few days after the implantation of tumor cells.

The situation is slightly more encouraging in the case of micrometastases. A significant reduction in the metastatic load has been documented with several cytokines. Once again, however, only a minority of the mice are cured.

A similar picture is emerging from phase I studies of the vaccination of cancer patients with transduced human tumor cells (Table 2 and Figure 1). The approach itself is safe, but the earliest available results show that only about 10% of patients displayed an objective response [55–59]. The immunological performance status of phase I patients was obviously suboptimal for this type of

Table 3. Therapeutic efficacy of combined cytokine gene transfer against established, non-engineered rodent tumors [61–85].

Genes	Number of protocols	Percentage of protocols reporting therapeutic efficacy[a]				Additive effects[b]
		Null	Low	Intermediate	High	
IL-2 + B7.1	1				100%	100%
IL-2 + MAA	1		100%			100%
IL-2 + TK	3		67%		33%	100%
IL-2 + TK + GM-CSF	2			100%		100%
IL-2 + IFN-γ	4		25%	25%	50%	33%
IL-2 + IL-4	2		50%		50%	100%
IL-2 + MHC	1		100%			100%
IL-6 + CD	1				100%	NR
IL-7 + TK	1	100%				0%
IL-7 + TK + DC	1			100%		100%
IL-12 + B7.1	1		100%			0%
GM-CSF + IL-4	2			50%	50%	50%
GM-CSF + TNF-α	1		100%			0%
GM-CSF + B7.2	1		100%			100%
GM-CSF + IFN-γ	2		100%			100%
IFN-α + TK	4		50%		50%	100%
IFN-γ + IFN-α	2	50%		50%		0%
IFN-γ + alloMHC	1				100%	100%
IFN-γ + CD	2			50%	50%	100%
IFN-γ + IL-4	1			100%		100%
IFN-γ + TNF	1		100%			NR
Total	35	6%	40%	23%	31%	72%

Abbreviations: allo – allogeneic; CD – cytosine deaminase; DC – dendritic cells; GM-CSF – granulocyte macrophage colony stimulating factor; IFN – interferon; IL – interleukin; MAA – melanoma associated antigen; MHC – class I major histocompatibility complex; NR – not reported; TK – thymidine kinase; TNF – tumor necrosis factor.

[a] Therapeutic efficacy was classified as: low when < 25% of mice were tumor and metastasis free; intermediate when tumor and metastasis free mice were between 25% and 50%; high when > 50% of mice were tumor and metastasis free.

[b] Percentage of protocols reporting significantly higher efficacy for combination vs. single genes.

therapy. Even so, one would have expected a greater number of responders in support of the potential promise of gene immunotherapy as a new form of treatment.

One is reminded of the early days of chemotherapy. A significant degree of therapeutic success evidently requires a switch from single-gene protocols to multiple-gene therapy.

From single-gene therapy to multiple-gene therapy

Several recent studies have reported greater therapeutic activity by vaccines made of tumor cells transduced with multiple genes (Table 3). A marked improvement as compared to single-gene vaccines was usually apparent, and up to 80%–100% of animals were cured by multiple-gene vaccines. We believe this to be the correct approach and that clinical studies along these lines will produce a sizeable proportion of objective responses.

Open issues in multiple-gene therapy are selection of the molecules to be combined in a protocol, and the schedule of administration.

The most complicated question is which cytokine genes should be combined to obtain the most effective therapeutic vaccine, a daunting prospect given the huge number of possible combinations. Two simple concepts can be borrowed from 'conventional' antineoplastic pharmacology to help bring the problem into clearer focus. First, we should test only those genes that made effective vaccines as single agents. Here the antimetastatic activity will provide the best quantitative estimate of the relative potency of various genes. Second, priority should be given to combinations of genes coding for molecules with independent mechanisms of action to maximize the probability of discovering synergistic combinations.

The dosage of cytokine received by the host following administration of cytokine gene-transduced cells is a non-standardized variable, in most cases a completely unknown quantity. The range of genetic engineering systems currently in use, and the obvious differences between tumors, explain why the *in vitro* release of cytokines by tumor cells used for therapeutic protocols spans several orders of magnitude, and comparisons are very difficult. After *in vivo* implantation the quantitation of local cytokine release by engineered tumor cells is practically impossible. The immune response elicited in the host is a more meaningful indicator of bioactive cytokine release *in vivo*, and further efforts should therefore be dedicated to the validation of immunological tests that could reliably measure vaccine activity in both experimental and clinical situations, and predict therapeutic activity.

Timing is a big problem insufficiently addressed by current preclinical studies. 'The sooner the better' is certainly a good rule of thumb for the starting of therapeutic vaccinations, but how do we proceed in the case of multiple-gene transduced cells? All that can be said is that the current preclinical models are inadequate for investigation of timing. The fastest murine models allow the evaluation of antimetastatic therapies within three weeks after the intravenous challenge to the appearance of macroscopic lung nodules. One day thus corresponds to 5% of the entire natural history of this development, whereas the time needed to mount a full-fledged secondary immune response is roughly equivalent to the entire natural history of metastases. We now need models with time frames better capable of accommodating the assessment of alternating regimens. One possible suggestion is the use of spontaneous tumors in transgenic mice carrying oncogenes, especially when they are accompanied by autochthonous metastases. Only appropriate preclinical models will allow the establishment of a rational basis for multiple-gene therapy.

The main difference between multiagent chemotherapy and multiple cytokine gene therapy is that the aim of the former is to hit the same cellular target with multiple bullets, while the objective of the latter is to orchestrate a multicellular response. The order of production of cytokines in a physiologic immune response is tightly orchestrated, and variations in timing of pro-

ductions or administration can have distinct biological effects. Moreover, physiological immune responses are self-limiting to prevent over-reaction to a particular stimulus. A number of inhibitory signals intervene in the late phases to switch off an episode of immune activation. Some feedback regulatory circuits are known in which the same cytokine or receptor plays an activatory role in the early phases of an immune response and an inhibitory role later on. Thus, succesful treatment strategies may entail both augmenting the stimulation of response and interfering with its physiological down-regulation.

It should also be noted that nearly all cytokines are pleiotropic, and the precise biological effects elicited *in vivo* by multiple cytokine gene therapy depends on host and tumor variables that will require a precise (and sometimes problematic) assessment, such as the degree of immune suppression, the type of pre-existing (spontaneous) immune response, intrinsic tumor immunogenicity, tumor interstitial pressure and vascularity, direct effects of cytokines on tumor cells, and many other things.

Next challenge: Integration of multiple-gene immunotherapy in the multidsciplinary approach to cancer

Some preclinical studies, including ours [86], have specifically assessed the toxicity of transduced cells and of the released cytokines. The only notable toxic effects were observed when the vaccine had a residual tumorigenicity leading to the release of pharmacological doses of the cytokine, or when critical injection sites were used, e.g., intracranial administration of cells releasing retroviruses [32]. Clinical phase I trials have confirmed that genetically engineered vaccines are generally safe [59].

We thus do not envisage any conceptual obstacle to the integration of genetically engineered vaccines with promising preclinical activity in future adjuvant trials, especially when vaccinations are scheduled during the long observation periods following chemo- and radiotherapy.

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DNA Vaccination Against Rat Her-2/Neu p185 More Effectively Inhibits Carcinogenesis Than Transplantable Carcinomas in Transgenic BALB/c Mice[1,2]

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The ability of vaccination with plasmids coding for the extracellular and the transmembrane domain of the product of transforming rat Her-2/*neu* oncogene (r-p185) to protect against r-p185$^+$ transplantable carcinoma (TUBO) cells and mammary carcinogenesis was evaluated. In normal BALB/c mice, DNA vaccination elicits anti-r-p185 Ab, but only a marginal CTL reactivity, and protects against a TUBO cell challenge. Massive reactive infiltration is associated with TUBO cell rejection. In BALB/c mice transgenic for the rat Her-2/*neu* gene (BALB-neuT), DNA vaccination elicits a lower anti-r-p185 Ab response, no CTL activity and only incompletely protects against TUBO cells, but markedly hampers the progression of carcinogenesis. At 33 wk of age, when control BALB-neuT mice display palpable tumors in all mammary glands, about 60% of immunized mice are tumor free, and tumor multiplicity is markedly reduced. Tumor-free mammary glands still display the atypical hyperplasia of the early stages of carcinogenesis, and a marked down-modulation of r-p185, along with a massive reactive infiltrate. However, BALB-neuT mice protected against mammary carcinogenesis fail to efficiently reject a TUBO cell challenge. 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 Journal of Immunology,* **2000, 165: 5133–5142.**

The Her-2/*neu* gene encodes a p185 tyrosine kinase growth factor receptor homologous to other members of the epidermal growth factor receptor family (1). Overexpressed or mutated p185 leans toward the formation of homo- or heterodimers with other epidermal growth factor receptor. As these dimers transduce positive growth signals in a ligand-independent way (2), they are involved in the initiation and progression of neoplastic transformation (2, 3).

Overexpression of p185 is frequent in human cancers and correlates with particular aggressiveness (4). In the rat, a single point mutation that replaces the valine residue at position 664 in the transmembrane (TM)[4] domain of p185 with glutamic acid favors p185 homo- and heterodimerization and transforms the Her-2/*neu* protooncogene into a dominant transforming oncogene (5). No such mutation, but only an increased Her-2/*neu* gene copy number and/or excess cell membrane expression of p185, was characterized in human tumors (6). However, recent detection in human breast cancer cells of alternatively spliced forms of Her-2/*neu* that resemble transforming Her-2/*neu* suggests that alternative splicing or mutations may also play a critical role in the development of human cancer (7–9).

The involvement of overexpressed and mutated p185 in the initiation and progression of breast carcinogenesis makes it an interesting target for therapy (10–12) and a docking site for toxins (13) and Ab (14). Normal and rat Her-2/*neu* transgenic mice immunized in various ways to Her-2/*neu* display a protective immune response against transplantable Her-2/*neu* tumors and their artificial metastases (15, 16). Moreover, both specific (17–19) and nonspecific (20, 21) immune reactions elicited in mice transgenic for rat Her-2/*neu* protooncogene and transforming oncogene are variously capable of hindering the development of mammary tumors.

In this paper, we assessed whether DNA vaccination with plasmids coding the TM and extracellular domain (ECD) of the rat p185 (r-p185) elicits a protective immune response. In normal BALB/c mice, r-p185 is a xenogeneic protein, even if mouse p185 and r-p185 differ in <6% of the amino acid residues (22). DNA vaccination elicits complete protection against a lethal challenge of syngeneic carcinoma cells expressing the r-p185 (TUBO cells). In BALB/c mice transgenic for the transforming rat Her-2/*neu* oncogene (BALB-neuT mice), r-p185 is a self-protein. DNA vaccination elicits an incomplete protection against TUBO cells, whereas it protects a significant number of mice against the aggressive progression of the carcinogenesis that takes place in all their mammary glands. An anti-r-p185$^+$ CTL response was never found in these mice, whereas they display a significant titer of anti-r-p185

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[4] Abbreviations used in this paper: TM, transmembrane; β-gal, β-galactosidase; ECD, extracellular domain; neuT, Her-2/*neu*-mutated transforming oncogene; PMN, polymorphonuclear leukocytes; r, rat; rV-neu, recombinant vaccinia virus expressing the r-p185; Spc, spleen cells; TSA-pc, TSA parental cells; V-wt, wild-type Wyeth virus; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage-inflammatory protein-2.

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Ab that may be responsible for the down-modulation of r-p185 observed in preneoplastic mammary cells. However, a TUBO cell challenge is still able to grow progressively in half of the BALB-neuT mice whose mammary carcinogenesis is fully inhibited. This finding suggests that the mechanisms required for the rejection of transplantable tumors may not fully coincide with those that inhibit the slow progression of carcinogenesis and that preneoplastic lesions are particularly sensitive to immune mechanisms elicited by DNA vaccination. It also offers support for the use of DNA vaccination as a new approach in the prevention of tumors expressing oncogenic growth factor receptors on their membrane.

Materials and Methods

Mice

Inbred BALB/c mice overexpressing the transforming rat Her-2/*neu* oncogene ($neuT^+/neuT^-$) driven by the mouse mammary tumor virus promoter (BALB-neuT) and transgene negative ($neuT^-/neuT^-$) (BALB/c) were produced and screened for the presence of the transgene as previously described in detail (20). Groups of individually tagged virgin BALB-neuT and BALB/c females bred under specific pathogen-free conditions by Charles River Breeding Laboratories (Calco, Italy) were treated in accordance with European Union and institutional guidelines. Since all 10 mammary glands of BALB-neuT females undergo carcinogenic transformation with a definite progression (20), these were inspected weekly, and tumor masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, at which time mice were sacrificed for humane reasons. Except where otherwise specified, surviving BALB-neuT mice were sacrificed at 33 wk (20). Because some immunized mice do not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas per mouse was calculated as the cumulative number of incident tumors/total number of BALB-neuT mice.

DNA expression vectors and vaccination

The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A). The sequence for the ECD and that for the ECD and TM domain of mutated r-p185 were generated from the PCR product using the primers 3′-CGCAAGCTTCATCATGGAGCTGGC-5′ and 3′-CGGAATT CGGGCTGGCTCTCTGCTC-5′ and the primers 3′-CGCAAGCTTCAT GGAGCTGGC-5′ and 3′-ATGAATTCTTTCCGCATCGTGTACTTCT TCCGG-5′, respectively, as previously described (19). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the two plasmids used in this work (ECD and ECD-TM plasmids). The pCMVβ (Clontech Laboratories, Palo Alto, CA) coding for β-galactosidase was used as a control plasmid (β-gal plasmid). *Escherichia coli* strain DH5α was transformed with ECD, ECD-TM, and β-gal plasmids and then grown in Luria-Bertani medium (Sigma, St. Louis, MO) (19). Large-scale preparation of the plasmids was conducted by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA). DNA was then precipitated, suspended in sterile saline at the concentration of 1 mg/ml, and stored in aliquots at −20°C for subsequent use in immunization protocols. Plasmids (100 μg/injection) were injected into the quadriceps muscle through a 28-gauge needle syringe. BALB/c mice were immunized 21 and 7 days before tumor challenge (day 0), starting at the 10th wk of age. BALB-neuT mice were immunized at the 10th and 12th wk of age or at the 6th, 12th, 18th, and 24th wk of age.

Cell lines and recombinant vaccinia virus

TUBO cells are a cloned cell line established in vitro from a lobular carcinoma that arose spontaneously in a BALB-neuT mouse. TSA parental cells (TSA-pc) are an aggressive and poorly immunogenic cell line established from a moderately differentiated mammary adenocarcinoma that spontaneously arose in a BALB/c mouse from Charles River Breeding Laboratories (23). F1-F is a newborn BALB/c mouse-derived skin fibroblast line spontaneously transformed after the 15th in vitro passage (24). Both TUBO and NIH3T3 (American Type Culture Collection, Manassas, VA) cells were cultured in DMEM (BioWhittaker Europe, Verviers, Belgium) supplemented with 20% and 5% FBS (Life Technologies, San Giuliano Milanese, Italy), respectively; TSA-pc and F1-F cells were cultured in RPMI 1640 (BioWhittaker Europe) with 10% FBS.

Flow cytometry

The expression of r-p185 was evaluated by using 7.16.4 mAb (Oncogene Research Products, Cambridge, MA). mAb against H-$2K^d$ (clone 31-3-4S) H-$2D^d$ and Ia^d (clone 28-16-8S) were obtained from Cederlane (Hornby, Ontario, Canada). Cells were stained in a standard indirect immunofluorescence procedure with primary Ab followed by a fluorescein-conjugated anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, MD). Cells were resuspended in PBS containing 1 mg/ml propidium iodide to gate out dead cells and evaluated in a FACScan (Becton Dickinson, Mountain View, CA). Flow cytometry showed that TUBO cells are highly positive for r-p185 and class I glycoproteins of the MHC. TSA-pc and F1-F cells express MHC class I, but not class II glycoproteins (23, 24) nor r-p185. To evaluate the presence of Ab capable of binding TUBO cells, sera were collected from six tumor-free BALB-neuT mice before (at 6 wk of age) or after (at wk 33) ECD-TM DNA immunization. A total of 2×10^5 TUBO cells was stained in a standard indirect immunofluorescence procedure with 50 μl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera followed by a fluorescein-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark). The cells were resuspended in PBS-azide-BSA containing 1 mg/ml propidium iodide and evaluated using a FACScan (Becton Dickinson).

Cytotoxicity assays

The CTL activity of lymphocytes from the mice of the various groups was independently tested immediately or after in vitro restimulation in the laboratory of G.F. (Torino, Italy), M.P.C. (Milan, Italy), and P.-L.L. (Bologna, Italy). Lymphocytes (1×10^7) were stimulated for 6 days with 5×10^5 irradiated TUBO cells as described previously (24). To get better stimulation, this basic design was variously changed in the several repeats of the test. Other rat Her-2/*neu* expressing BALB/c target cells were also used as stimulator and target cells. Moreover, the suppressor activity of stimulator rat Her-2/*neu* BALB/c cells was ruled out by adding progressive numbers of third-party TUBO cells in mixed lymphocyte and allogeneic target cell interactions as previously described (25). CTL activity of fresh and restimulated lymphocytes was assayed in 4- and 18-h ^{51}Cr release assays in Milan (26), in 48- and 72-h [^{3}H]TdR release assays in Torino (24, 25), and in 18- and 40-h [^{3}H]proline release assays in Bologna (27) as previously described in detail. In all of these tests, both TUBO cells and other rat Her-2/*neu* expressing BALB/c target cells were highly lysable by allogeneic CTL.

Cell internalization of r-p185

Expression of p185 was evaluated by confocal microscopy. A total of 2×10^5 TUBO cells was suspended in DMEM, incubated with 50 μl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera for 3 h at 4°C or at 37°C, and washed twice with cold PBS-azide-BSA. For detection of cytoplasmic r-p185, TUBO cells were incubated with 1 ml of PBS-4% paraformaldehyde. After 20 min at 4°C, TUBO cells were washed twice with cold PBS-azide-BSA and then incubated with 1 ml of PBS-0.3% Triton X-100. After 30 min at room temperature, TUBO cells were washed twice with cold PBS-azide-BSA. Membrane and cytoplasmic expression of r-p185 TUBO cells was assessed by staining with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Internalization of fluorescent mAb was then measured on a confocal microscope (LFM310; Zeiss, Jena, Germany) (488-nm argon laser and 543-nm helium-neon laser). Green fluorescence was detected after excitation at 488 nm. Images were recorded as TIF files and processed (LSM Image Examiner; Zeiss) to subtract background and enhance lower and middle intensity fluorescence.

Tumor challenge and evaluation of tumor growth

At the times specified, mice were challenged s.c. in the left flank or in the neck region with 0.2 ml of a single-cell suspension of 1×10^5 TUBO or TSA-pc cells or 1×10^4 F1-F cells. These are about the minimal 100% tumor-inducing doses in BALB/c mice (24). The same minimal 100% tumor-inducing dose for TUBO cells was found in both BALB/c and BALB-neuT mice. Subcutaneous neoplastic masses were measured with calipers in two perpendicular diameters. The cages were coded, and the incidence and growth of tumors were evaluated weekly for 60 days in a fashion blind to the group in which they had been treated. Mice tumor free at the end of this period were classed as survivors. Neoplastic masses were measured with calipers in the two perpendicular diameters. Mice with a tumor mass with a mean diameter of >3 mm were classed as tumor bearers. Mice bearing neoplastic masses of >10 mm in mean diameter were killed for humane reasons.

Winn assay

The inhibition of TUBO cell growth in vivo was assayed using the Winn-type neutralization assay as previously described in detail (28). Various numbers of nylon-wool column-purified spleen cells (Spc) were admixed with the minimal lethal dose of TUBO or TSA cells in 0.2 ml of PBS and immediately injected s.c. in the left inguinal region of recipient mice. The ratios of lymphocytes:tumor cells were 1:1, 5:1, and 20:1.

Morphological analysis

Groups of three BALB-neuT mice were sacrificed at the indicated times each week until the 33rd wk. For histological evaluation, tissue samples were fixed in 10% neutral-buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with anti-dendritic cells (NLDC 145; Cederlane), anti-CD4 and anti-CD8a (Sera-Lab, Crawley Down, Sussex, U.K.), anti-Mac-1 (anti-CD11b/CD18), anti-Mac-3 and anti-Ia (Boehringer Mannheim, Milan, Italy), anti-polymorphonuclear leukocytes (PMN) (RB6-8C5, provided by R. L. Coffman, DNAX, Palo Alto, CA), anti-asialo GM1 (Wako Chemicals, Dusseldorf, Germany), anti-endothelial cells (mEC-13.324), anti-ELAM-1 (E-selectin; both provided by A. Vecchi, Istituto M. Negri, Milan, Italy); anti-ICAM-1 (CD54), anti-VCAM-1 (PharMingen, San Diego, CA), anti-IL-4, anti-IL-6, anti-IL-10, anti-IL-12, and anti-monocyte chemoattractant protein-1 (MCP-1) (PharMingen); anti-macrophage-inflammatory protein-2 (MIP-2; Walter Occhiena Srl, Torino, Italy); anti-RANTES (Pepro-Tech, Rocky Hill, NJ); anti-IL-1β (Genzyme, Cambridge, MA), anti-TNF-α (Immuno Kontact, Frankfurt, Germany), anti-IFN-γ (provided by S. Landolfo, University of Turin, Torino, Italy), and anti-inducible NO synthase (Transduction Laboratories, Lexington, KY) Ab. To evaluate the expression of r-p185 Ag, paraffin-embedded sections were tested with anti-neu Ab (C-18)-G (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, they were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Ig (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ab was removed by washing and the slides were incubated with ABC complex/AP (Dako). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blind fashion. From mice with multiple tumors, one sample per tumor growth area and 10 randomly chosen fields in each sample were evaluated for each point determination. Positive cells were counted under a microscope ($\times$400 field, $\times$40 objective, and $\times$10 ocular lens; 0.180 mm^2/field). The expression of adhesion molecules, cytokines, and mediators was defined as absent (−), scarcely (+/−), moderately (+), and frequently (++) present on cryostat sections tested with the corresponding Ab.

ELISA titration of anti-r-p185 Ab

Serum samples were titrated for the presence of anti-r-p185 Ab by ELISA and immunoprecipitation followed by Western blot analysis as described previously (29). NIH3T3 fibroblasts were infected with the V-Wyeth virus (wild-type control virus, V-wt) or with the recombinant vaccinia virus expressing the r-p185 (rV-neu), both kindly provided by Therion Biologics (Cambridge, MA). The r-p185 recombinant protein was detected by Western blot analysis using the Ab-1 polyclonal Ab at 1 mg/ml as described below. For ELISA, 5×10^4 NIH3T3 cells/well were allowed to adhere overnight in 96-well culture plates. After washing with PBS, sucrose gradient-purified rV-neu and V-wt viruses were added for 12–18 h at 10 PFUs/well. Plates were then dried overnight and nonspecific binding was blocked by a 1-h incubation with 5% BSA in PBS. Ab-4 mAb or mouse myeloma protein (MOPC21; Cappel, West Chester, PA) at 1 μg/ml or mouse serum pool (1:5/1:50/1:250/1:1250 dilutions) were added for 3 h at 30°C. After washing, HRP-conjugated goat anti-mouse IgG plus IgM (Life Technologies, Rockville, MD) was added, and the bound Ab was detected with *o*-phenylenediamine dihydrochloride (Sigma). The reaction was stopped with 25 μl of 4 N H_2SO_4, and the absorbance at 492 nm was evaluated with an automatic ELISA reader. The specific absorbance of each sample was calculated by subtracting its absorbance from that of V-wt NIH3T3 cells. The titer of the serum was defined as the highest dilution reaching a specific binding with an OD of 0.3. The contribution of each isotype to the anti-r-p185 titer was evaluated using a 1:500 and a 1:50 dilution of BALB/c and BALB-neuT sera, respectively, and a sera Mouse Typer Isotyping kit (Bio-Rad, Richmond, CA) as previously described (30). The percentage was calculated as the ratio of the specific OD 492-nm values of each isotype and that of all isotypes $\times$100.

Western blots

For immunoprecipitation, NIH3T3 cells were infected with 10 PFU/cell of either V-wt or rV-neu and cultured at 37°C for 18 h. Cell lysates were prepared in lysis buffer (10 mM sodium phosphate (pH 7.4), 100 mM NaCl, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) containing 100 μg/ml aprotinin and 1 mM PMSF. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad) according to Bradford (31).

One microgram of Ab-4 mAb or purified MOPC-21 mouse myeloma protein or 3 ml of mouse serum and 20 μl of protein G-Sepharose were reacted with 300 μg lysate of V-wt- or rV-neu-infected NIH3T3 cells for 3 h at 4°C. The beads were washed with Staph A buffer, and the pellets were denatured by boiling for 5 min in 30 μl of sample buffer (100 mM Tris (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 50 mM 2-ME). Electrophoresis of immunoprecipitates or protein lysates (100 μg/lane) was conducted in denaturing 8% Tris-glycine polyacrylamide gels (SDS-PAGE). Gels were then processed for immunoblotting using Ab-1 polyclonal Ab at 1 μg/ml and bound Ab were visualized as previously described (30).

Statistical analysis

Differences in tumor incidence were evaluated using the Mantel-Haenszel log rank test, differences in tumor/mouse numbers using Wilcoxon's rank sum test, and differences in the number of tumor-infiltrating cells by Student's *t* test.

Results

r-p185 expressed by TUBO cells is poorly immunogenic in normal BALB/c mice

TUBO cells are a cloned line established in vitro from a BALB-neuT mouse mammary carcinoma. They display membrane class I H-2^d MHC glycoproteins and r-p185 neu proteins (Fig. 1, *upper panels*). In BALB/c mice, r-p185 is a xenogeneic Ag that differs from mouse r-p185 in <6% of the amino residues (22). Despite these differences, a challenge of 1×10^5 TUBO cells grew progressively in all BALB/c mice (Table I) and gave rise to lobular carcinomas histologically similar to those that appear in BALB-neuT-transgenic mice (Figs. 1*e* and 2*c*). The reactive cell infiltrate associated with TUBO cell growth was marginal and not stronger than that of the fully syngeneic TSA-pc mammary carcinoma (Table II). No anti-TUBO cell CTL, nor IFN-γ, nor GM-CSF release were found when Spc from BALB/c mice bearing 3- or 10-mm mean TUBO tumors were tested immediately or after 6 days in in vitro restimulation with TUBO cells as previously described in detail (24, 25) (data not shown). Moreover, no anti-r-p185 Ab were detected in the sera of mice receiving saline or immunized with β-gal plasmid (Fig. 3). Despite their high membrane expression of the xenogeneic r-p185, growing TUBO cells appear to trigger a marginal or no immune reaction in BALB/c mice.

DNA vaccination induces protective immunity against TUBO cells in BALB/c mice

A significant and specific inhibition of TUBO cells was found in BALB/c mice immunized with either ECD or ECD-TM plasmids 21 and 7 days before TUBO cell challenge (Table I). TUBO cells initially formed small cell aggregates infiltrated by reactive leukocytes in close contact with severely injured tumor cells (Fig. 1*h*). Rejection was associated with a marked influx of PMN, $CD4^+$ and $CD8^+$ T lymphocytes, and an increase in the number of dendritic cells, macrophages, and NK cells (Table II). Induction and increased expression of endothelial cell adhesion molecules was also evident in tumor vessels. IFN-γ and MCP-1 were expressed, while overexpression of IL-1β, TNF-α, and IL-10 was also evident. No TUBO cell inhibition was found in BALB/c mice injected with saline only or immunized with β-gal plasmids. Since the results from these two treatment groups were consistently similar, hereafter both groups are cumulatively denominated as controls.

Anti-r-p185 Ab were detected in the sera of both ECD- and ECD-TM-immunized mice. They are mainly IgG2a, IgM, and IgG1 (Fig. 3). All sera pools scored positive immunoprecipitated

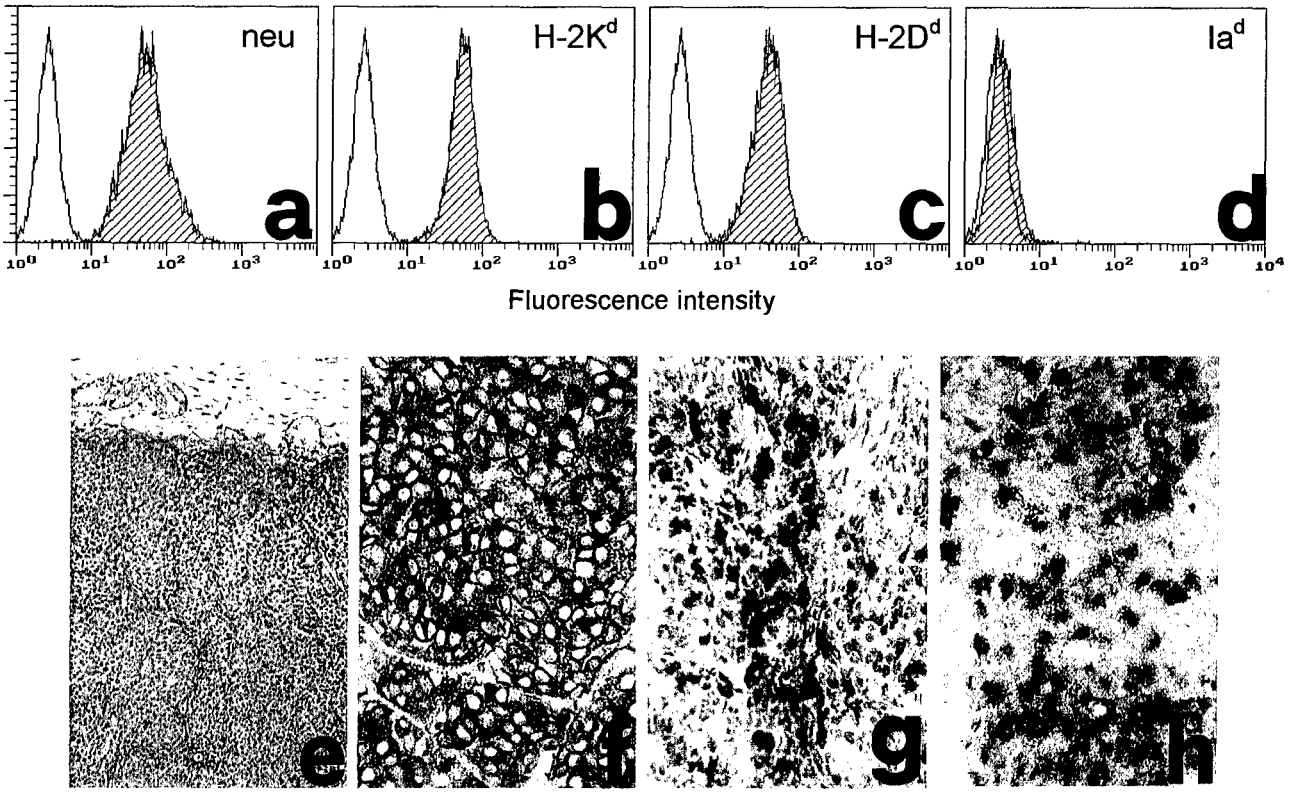

FIGURE 1. Features of TUBO cells. *Upper panels*, Flow cytometric analysis of the expression r-p185 protein (*a*), MHC class I (H-2K^d, *b*; H-2D^d, *c*), and MHC class II (Ia^d; *d*) by TUBO cells. Open profiles, Cells stained with secondary Ab; shaded profiles, cells stained with anti-r-p185 or anti-MHC Ab. In each graph, the ordinate represents the number of cells and the abscissa reports the fluorescence intensity in logarithmic arbitrary units. *Lower panels*, TUBO cells injected s.c. in BALB/c mice gave rise to lobular carcinomas histologically similar to the mammary carcinomas arising in BALB-NeuT mice (*e*) that homogeneously express r-p185 on their cell membrane (*f*). TUBO cell rejection area in BALB/c mice immunized with ECD-TM plasmid was heavily infiltrated by CD4$^+$ T lymphocytes (*g*) and PMN (*h*). Original magnification: *e*, ×200; *f–h*, ×630).

the r-p185 from the NIH3T3 cells infected with rV-neu and not those with V-wt virus. Those scored negative did not (data not shown). The anti-r-p185 Ab titer did not substantially increase after a subsequent challenge and rejection of TUBO cells. In contrast with both the Ab response and the marked cell reaction at tumor site, a marginal CTL activity and no IFN-γ release against TUBO cells were found in Spc from ECD- or ECD-TM-immunized BALB/c mice collected 7 days after the last immunization and tested immediately or after 6 days in in vitro restimulation with TUBO cells (data not shown). Moreover, both fresh and in vitro restimulated Spc did not block TUBO cell growth in a Winn assay (28), and only a slight delay of tumor growth was found at a 20:1 lymphocyte:tumor cell ratio.

DNA vaccination induces a partial protection against TUBO cells in BALB-neuT mice

In female BALB-neuT mice, r-p185 is a self-protein markedly expressed in terminal ductal-lobular structures of the mammary glands as early as the third week of age (Fig. 2*a*). To evaluate whether DNA vaccination induces a protective response to TUBO cells, BALB-neuT mice were immunized with the ECD-TM plasmid two or four times. Vaccination on week 10 and 12, when hyperplasia of the terminal ductal-lobular structures is already evident, protects a few mice against a TUBO challenge 7 days after the second immunization (Table III). However, the latency of the tumors that eventually grew was extended in immunized as compared with control mice. Partial protection, too, was found in mice immunized four times and challenged with TUBO cells at 33 wk of age (Table III). Immunohistochemistry showed that the tumor area of the TUBO challenge in ECD-TM-immunized mice presents a significant increase in the number of CD8$^+$ cells and PMN as compared with control mice (Table II). No difference in the expression of endothelial adhesion molecules, cytokines, and mediators was found.

No anti-r-p185 Ab were found in the sera from mice challenged with TUBO cells only or immunized with saline or β-gal plasmids. The titer of those found in sera from ECD-TM-immunized mice was higher in animals that received four vaccinations (Table IV).

DNA vaccination effectively halts carcinogenesis in BALB-neuT mice

Since DNA vaccination elicited a partial resistance against TUBO cells, its ability to hamper the aggressive carcinogenesis that takes

Table I. *Growth and rejection of TUBO cells in control, ECD-immunized, and ECD-TM-immunized BALB/c mice*

BALB/c Mice Immunized with[a]	Tumor Take/Mice Challenged with		
	TUBO cells	TSA-pc	F1-F cells
Saline only	12/12 (0%)[b]	6/6 (0%)	6/6 (0%)
β-gal plasmid	6/6 (0%)	6/6 (0%)	6/6 (0%)
ECD plasmid	0/6[c] (100%)	ND	6/6 (0%)
ECD-TM plasmid	0/12[c] (100%)	6/6 (0%)	6/6 (0%)

[a] Performed at days −21 and −7 before challenge (day 0).
[b] Percentage of survival in parentheses.
[c] $p \leq 0.001$ as compared to BALB/c mice receiving saline only.

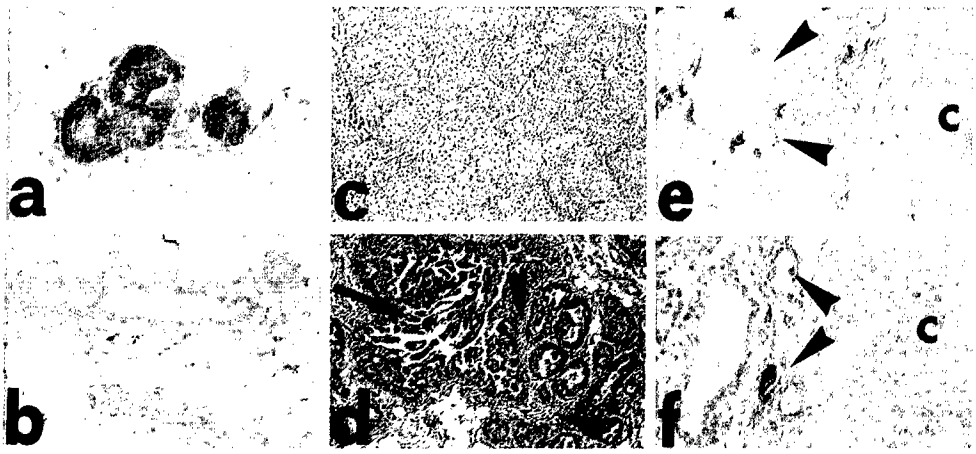

FIGURE 2. Morphological events associated with the inhibition of carcinogenesis in BALB-neuT mice. r-p185 is markedly expressed (in brown) in the hyperplastic terminal ductal-lobular structures of the mammary glands of females 3-wk-old BALB-NeuT mice (*a*). At 33 wk of age, all mammary glands of control BALB-NeuT mice show invasive lobular carcinomas (*c*), whereas several glands from ECD-TM-immunized mice display areas composed of numerous ductules lined by a monolayer of epithelial cells without membrane or cytoplasmic r-p185 expression (*b*). Serial sections indicate that the majority of these ductules do not end in differentiated terminal ductal-lobular structures. Some glands show areas of atypical hyperplasia with the interposed stroma (arrowheads) markedly infiltrated by reactive cells and foci of desegregated lobular carcinoma (arrow) with loosely cohesive tumor cells (*d*). Hyperplastic areas are infiltrated by $CD8^+$ lymphocytes (arrowheads, *e*) while microvessels expressed VCAM-1 adhesion molecule (arrowheads, *f*). When areas of hyperplasia (*left*) and lobular carcinoma (*c*, *right*) are present in the same gland (*e* and *f*), infiltrating $CD8^+$ lymphocytes (*e*, arrowheads) and VCAM-1 expression (*f*, arrowheads) are evident in hyperplastic foci and almost absent in carcinomas. Original magnification: *a*, *b*, *e*, and *f*, ×400; *c* and *d*, ×200.

place in all of the mammary glands of BALB-neuT mice was assessed. Mice were immunized at the 6th, 12th, 18th, and 24th wk of age with the ECD-TM plasmid, and then inspected weekly to follow tumor onset and growth. At 33 wk, when all 10 mammary glands of control mice presented an evident palpable mass, 57% of the immunized mice were still completely free (Fig. 4, *upper panel*). A significant reduction in tumor multiplicity was also evident (Fig. 4, *bottom panel*).

Pathological observations showed that at 33 wk of age control mice uniformly display invasive lobular carcinomas in all 10 glands (Fig. 2*c*). By contrast, three distinct patterns were displayed by the glands from ECD-TM-immunized mice. Those without a palpable mass showed numerous ductules lined by a monolayer of epithelial cells. In about 60% of cases, serial sections displayed truncated ductules that did not end in differentiated terminal ductal-lobular structures (Fig. 2*b*), and the remaining structures

Table II. *Reactive cell content, expression of endothelial adhesion molecules and production of cytokines and mediators at the tumor area 7 days after TSA-pc or TUBO challenge of BALB/c and BALB-neuT mice immunized with ECD-TM plasmid*

	BALB/c Mice Challenged with		ECD-TM-Immunized BALB/c Mice Challenged with TUBO Cells	BALB-neuT Mice Challenged with TUBO Cells	ECD-TM-Immunized BALB-neuT Mice Challenged with TUBO Cells
	TSA-pc	TUBO cells			
Reactive cells[a]					
Dendritic cells	14 ± 4	ND	6 ± 1[b]	–	–
Macrophages	20 ± 4	20 ± 5	55 ± 15[b]	19 ± 5	14 ± 4
PMN	14 ± 4	7 ± 2	76 ± 19[b]	6 ± 2	14 ± 3[b]
$CD8^+$ lymphocytes	5 ± 2	9 ± 3	30 ± 6[b]	4 ± 2	8 ± 2[c]
$CD4^+$ lymphocytes	ND	ND	40 ± 9[b]	1 ± 1	3 ± 2
NK cells	ND	10 ± 3	30 ± 5[b]	7 ± 2	8 ± 3
Endothelial adhesion molecules					
ICAM-1	+[d]	+	++	+	+
ELAM-1	–	–	+	+/–	+
VCAM-1	–	–	+/–	–	–
Cytokines and mediators					
IL-1β	–	+	++	+/–	+/–
TNF-α	+	+/–	+	+	+
IFN-γ	–	–	+	+/–	+/–
IL-4	–	–	–	ND	ND
IL-10	+	+	++	ND	ND
IL-12	–	–	–	ND	ND
MCP-1	–	–	+	–	–
MIP-2	+	+	+	+	+
RANTES	ND	ND	ND	+/–	+/–

[a] Cell counts performed at ×400 in a 0.180-mm^2 field. At least 3 samples (1 sample/tumor growth area) and 10 randomly chosen fields/sample were evaluated. Results are expressed as mean ± SD of positive cells/field evaluated on cryostat sections by immunohistochemistry.

[b] Values significantly different ($p < 0.001$) from corresponding values in untreated mice.

[c] Values significantly different ($p < 0.005$) from corresponding values in untreated mice.

[d] The expression of adhesion molecules, cytokines, and mediators was defined as absent (–), scarcely (+/–), moderately (+), and frequently (++) present on cryostat sections decorated with the Ab.

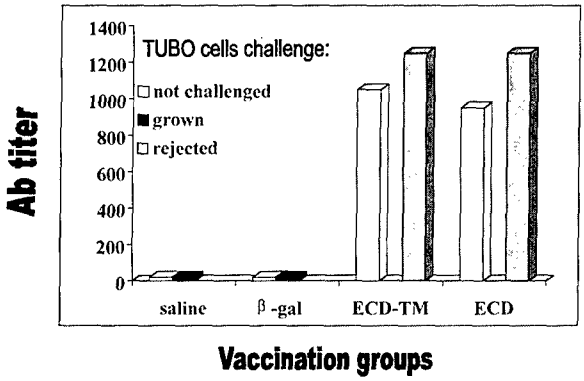

FIGURE 3. Titer of Ab to r-p185 in ECD- and ECD-TM-immunized BALB/c mice. Mice were immunized at days −21 and −7 before challenge (day 0), and sera collected at day 0 (before TUBO challenge) or 2 wk later. Sera from six mice were pooled and the titer was defined by ELISA as the reciprocal of the dilution reaching an OD of 0.3. The percentage of various isotype relative to the total anti-r-p185 Ig in the sera of ECD-TM-immunized mice that have rejected TUBO cells was: IgG2a, 59%; IgM, 26%; and IgG1, 12%.

showed a reduced tendency to give rise to foci of lobular atypical hyperplasia. Massive reactive cell infiltration of their stroma was associated with the induction and increased expression of IL-1β, TNF-α, and IFN-γ and of endothelial cell adhesion molecules (Table V).

Glands with no palpable tumors or with small tumor masses showed foci of in situ lobular carcinomas. In this case, however, the majority of tumor cells were desegregated and loosely cohesive (Fig. 2*d*). Reactive leukocytes and expression of proinflammatory cytokines were both more marked than in carcinomas of control mice and much less numerous than in the hyperplastic foci (Table V). These differences were also evident when both carcinoma and hyperplasia were present in contiguous areas of the same mammary gland (Fig. 2, *e* and *f*). This suggests that hyperplasia is open to the immune reactivity, but becomes much less susceptible once it has progressed to carcinoma. In both cases, reactive leukocytes were in the stroma surrounding hyperplastic and neoplastic lobules and did not penetrate the basal membrane (Fig. 2*d*). Finally, mammary glands with >4-mm mean diameter tumor masses displayed

Table III. *Growth and rejection of TUBO cells in control and ECD-TM-immunized BALB-neuT mice*

BALB-neuT Mice Immunized and Challenged			TUBO Cell Growth	
Vaccination plasmids	Week of vaccination	Week of TUBO cell challenge	Takes/ challenged mice	Latency time (days)
Saline only	10, 12	13	6/6 (0)[a]	22 ± 3
β-gal plasmid	10, 12	13	6/6 (0)	17 ± 1
ECD-TM plasmid	10, 12	13	4/6 (33%)	58 ± 17[b]
ECD-TM plasmid	6, 12, 18, 24	33	3/6 (50%)	20 ± 2

[a] Percentage of survival in parentheses.
[b] $p \leq 0.001$ as compared to BALB-neuT mice receiving saline only.

invasive carcinomas indistinguishable from control mice (data not shown).

Immune activities associated with the inhibition of carcinogenesis

Here, too, the marked infiltrate observed in pathological specimens did not correlate with the induction of a CTL response in Spc from immunized mice tested either fresh or after in vitro restimulation nor with their ability to inhibit the growth of TUBO cells in a Winn test (data not shown). By contrast, a significant anti-r-p185 Ab response, mainly represented by IgG2a, IgG1, IgM, and IgA (Table IV), was detected in sera from immunized mice. When incubated with TUBO cells, these sera induced stripping of r-p185 from the membrane and its cytoplasmic internalization (Fig. 5*g*). A similar impressive down-modulation of r-p185 membrane expression and its cytoplasmic expression only were observed in most hyperplastic lesions from ECD-TM-immunized mice (Fig. 5*h*). Their reduced r-p185 cell surface expression was accompanied by a diminished nuclear positivity of proliferating cell nuclear antigen (PCNA; data not shown). When the lesions progressed to carcinoma in situ, areas with high membrane expression of r-p185 and marked nuclear PCNA positivity alternated with others with only intracytoplasmic r-p185 and no PCNA expression (data not shown). No more r-p185 down-modulation but a similarly marked r-p185 membrane staining was evident in most neoplastic cells of invasive mammary carcinomas in immunized mice or in control BALB-neuT mice (data not shown).

Table IV. *Ab to r-p185 in ECD-TM-immunized BALB-neuT mice challenged with or without TUBO cells*

BALB-neuT Mice Immunized with	No. of Mice	Week of Vaccination	TUBO Cell Challenge	Growth of a TUBO Tumor (>3 mm diameter)	Week of Bleeding	Dominant Stage of the Mammary Glands	Titer of Ab to r-p185[a]
Saline	6	10, 12	NC[b]	−	13	Carcinoma in situ	<5
Saline	6	10, 12	Yes[c]	+	16	Carcinoma in situ	<5
β-gal plasmid	6	10, 12	NC	−	16	Carcinoma in situ	<5
β-gal plasmid	6	10, 12	Yes[c]	+	16	Carcinoma in situ	<5
β-gal plasmid	6	6, 12, 18, 24	NC	−	33	Invasive carcinoma	11
ECD-TM plasmid	4	10, 12	Yes[c]	+	24	Normal/Atypical hyperplasia	85
ECD-TM plasmid	2	10, 12	Yes[c]	−	24	Normal/Atypical hyperplasia	73
ECD-TM plasmid	4	6, 12, 18, 24	NC	−	33	Normal/Atypical hyperplasia	240[d]
ECD-TM plasmid	3	6, 12, 18, 24	NC	−	33	Atypical hyperplasia/Invasive carcinoma	200
ECD-TM plasmid	3	6, 12, 18, 24	Yes[e]	−	38	Atypical hyperplasia	200
ECD-TM plasmid	3	6, 12, 18, 24	Yes[e]	+	38	Atypical hyperplasia	230

[a] Ab titer was defined in ELISA as in Fig. 3.
[b] NC, Not challenged.
[c] Challenged with 1×10^5 TUBO cells at 13 wk of age.
[d] Ig isotypes of anti-r-p185 Ab: IgG2a, 32%; IgG1, 24%; IgM, 22%; and IgA, 13%.
[e] Challenged with 1×10^5 TUBO cells at 33 wk of age.

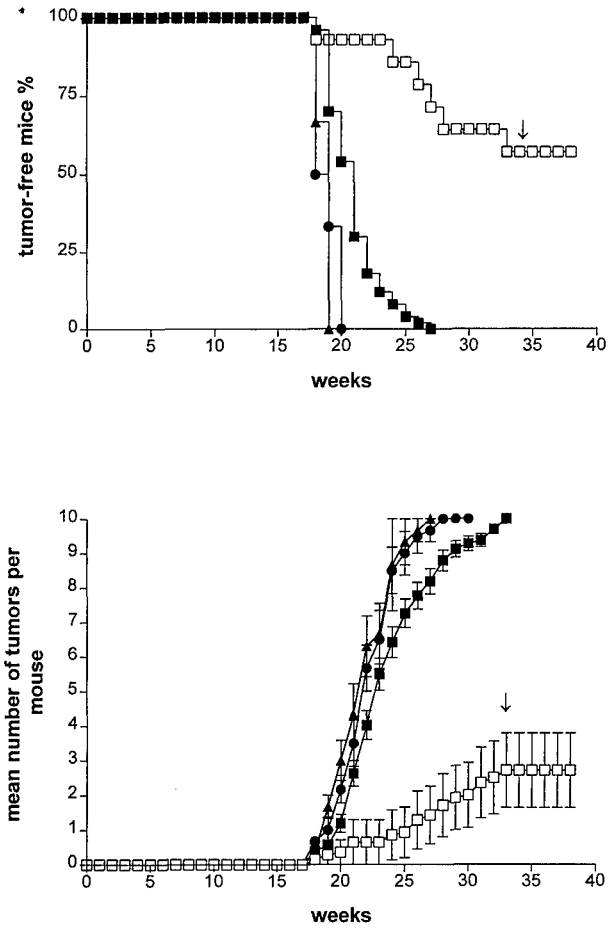

FIGURE 4. Effect of vaccination with ECD-TM plasmid on carcinogenesis in BALB-neuT mice. Time of appearance of the first tumor (*upper panel*) and mean number of palpable mammary carcinomas per mouse (*lower panel*) in the group of 50 untreated mice (■), of 6 mice treated with saline only (▲), and β-gal (●), and in a group of 14 mice immunized with ECD-TM (□). Arrow shows the week in which ECD-TM-immunized mice without any palpable tumors were challenged in the neck with TUBO cells.

BALB-neuT mice in which carcinogenesis is inhibited are still susceptible to TUBO cells

Six DNA-vaccinated BALB-neuT mice that did not display any palpable tumor at week 33 were challenged s.c. in the neck with TUBO cells (Fig. 4, arrow). Three rejected the challenge. In the other three TUBO cells grew after latency similar to the control BALB-neuT mice (Table III). No spontaneous mammary carcinoma progressed and became palpable in any of these six mice during the 35 days that followed the TUBO cell challenge, showing that inhibition of carcinogenesis can exceed 38 wk, when the mice were sacrificed.

Discussion

Present data show that vaccination with plasmids coding either ECD-TM or ECD of r-p185 protects BALB/c mice against a challenge with TUBO mammary carcinoma cells that express the xenogeneic r-p185 on their membrane. In transgenic BALB-neuT, r-p185 is a self-protein that is already overexpressed by the mammary gland at the third week of life. Nonetheless, vaccination with the ECD-TM plasmid protects a smaller, but significant portion of BALB-neuT mice against TUBO cells, and significantly inhibits the progression of their aggressive mammary carcinogenesis. At 33 wk, large lobular carcinomas are evident in all mammary glands of the controls (20, 32). At this time point, more than half of the immunized BALB-neuT mice are tumor free, and the mean number of glands with a palpable carcinoma is much lower than in the controls. Interestingly, not all mice whose carcinogenesis is fully inhibited reject a subsequent TUBO cell challenge.

These data show that DNA vaccination manages the aggressive progression of Her-2/*neu* mammary carcinogenesis more effectively than the nonspecific reactivity elicited by systemic IL-12 (20). The best IL-12 regimen markedly delays, but rarely inhibits this carcinogenesis (21). The mechanisms involved are also different. IL-12 induces the release of a series of downstream mediators that trigger nonspecific cellular immunity and impair the vascular proliferation associated with carcinogenesis (20, 33). Its antiangiogenic and angiotoxic activity is mostly effective on the fragile capillary sprouts associated with the shift from atypical hyperplasia to carcinoma (20). By contrast, ECD-TM vaccination results in inhibited progression unaccompanied by areas of ischemic-hemorrhagic necrosis or signs of vascular damage.

At 33 wk of age, three prototypic scenarios are displayed by the mammary glands of ECD-TM-immunized mice. Atypical hyperplasia is displayed by tumor-free mammary glands. In control BALB-neuT mice, this lesion connotes a much earlier phase of carcinogenesis. At variance with controls, the hyperplasia in immunized mice is accompanied by pronounced infiltration of dendritic cells, macrophages, PMN, and T lymphocytes and the local presence of proinflammatory cytokines. The high expression of adhesion molecules by vessel endothelial cells accounts for this marked reactive cell infiltration. Furthermore, the terminal ductal-lobular structures and hyperplastic foci were less prosperous, formed of epithelial cells that express cytoplasmic but not membrane r-p185, and displayed a reduced cell proliferation. In vitro, r-p185 stripping from TUBO cell membrane and its cytoplasmic internalization is induced by the Ab present in the sera of these mice.

Other mammary glands display evident in situ lobular carcinomas. By contrast with the much earlier corresponding lesions in the controls, carcinoma cells are desegregated and loosely cohesive and produce empty lacunar spaces. Areas with high membrane expression of r-p185 and marked nuclear PCNA positivity alternate with others with only intracytoplasmic r-p185 and no PCNA expression. Down-modulation of membrane r-p185 correlates with a restrained neoplastic proliferation. Finally, there were no pathological differences between the invasive carcinomas observed in the immunized mice and the controls. Although 38 wk is a significant period in the life of a mouse, the evidence on the presence of microscopic preneoplastic and neoplastic lesions in the mammary glands of vaccinated mice has spurred ongoing experiments to evaluate how long further boosting vaccinations inhibit the progression of carcinogenesis during the aging of mice.

Apparently both leukocyte infiltration and the release of proinflammatory cytokines decline as the hyperplasia progresses to carcinoma. The immune mechanisms elicited by ECD-TM vaccination reach hyperplastic lesions and halt their progression, but are incapable of dealing with an established carcinoma whose extracellular matrix (34, 35), neovessels (36), positive pressure (37), and release of many suppressive factors (38) secure its resistance to immune attack. These factors may also account for the poor ability of ECD-TM vaccination to inhibit TUBO cell challenges. TUBO cells, like transplantable tumors in general, very quickly give rise to solid tumor masses that are highly vascularized and refractory to most immune mechanisms (39).

The discrepancy between the impressive ability of immunized BALB-neuT mice to halt the progression to carcinoma and their relatively minor ability to inhibit the takes of transplantable TUBO

Table V. *Reactive cell content, expression of endothelial adhesion molecules, and production of cytokines and mediators in the mammary glands of control or ECD-TM-immunized BALB-neuT mice*

	Carcinomas in β-gal Immunized Mice (week 13)	Carcinomas in ECD-TM-Immunized Mice (week 33)	Atypical hyperplasia in ECD-TM-Immunized Mice (week 33)
Reactive cells			
Dendritic cells	1 ± 0^a	7 ± 2^b	10 ± 3^b
Macrophages	11 ± 3	22 ± 4^b	23 ± 4^b
PMN	3 ± 2	12 ± 3^b	$23 \pm 4^{b,c}$
$CD8^+$ lymphocytes	3 ± 1	8 ± 2^b	$30 \pm 6^{b,c}$
$CD4^+$ lymphocytes	2 ± 1	1 ± 0	$12 \pm 4^{b,c}$
NK cells	2 ± 1	5 ± 2^b	8 ± 2^b
Endothelial adhesion molecules			
ICAM-1	$+^d$	++	++
ELAM-1	−	+	++
VCAM-1	−	−	+
Cytokines and mediators			
IL-1β	−	+/−	+
TNF-α	+/−	+	++
IFN-γ	−	+/−	+
MCP-1	−	−	+/−
MIP-2	+/−	+	++

[a–d] As for Table II.

cells may be due to the type of reaction mechanisms activated by vaccination. ECD- and ECD-TM-vaccinated mice, in fact, never displayed a marked CTL response, despite all the in vitro restimulation attempts performed independently in three of the laboratories involved in this study. This provocative finding may suggest that in our system DNA vaccination was unable to break T killer cell tolerance against r-p185, a xenogeneic protein that shares >94% homology with mouse p185. This is somewhat surprising, since various peptides from r-p185 bind the grooves of $H\text{-}2^d$ class I and class II glycoproteins and display amino acid sequences different from those of mouse p185 (22). Despite the repeated efforts in CTL assessment, it is virtually impossible to rule out any potential flaws in our in vitro analyses. Moreover, the absence of detectable in vitro T cell activity does not necessarily correlate with an effective T cell-dependent antitumor immunity in vivo (28). In contrast, pathological findings indicated that reactive cells were in the stroma surrounding hyperplastic and neoplastic terminal ductal-lobular structures of the mammary, but they never penetrated the basal membrane and were found intermingled with neoplastic epithelial cells.

The only in vitro finding that correlates with the protection in vivo and the evidence of immune reactions at challenge sites or in

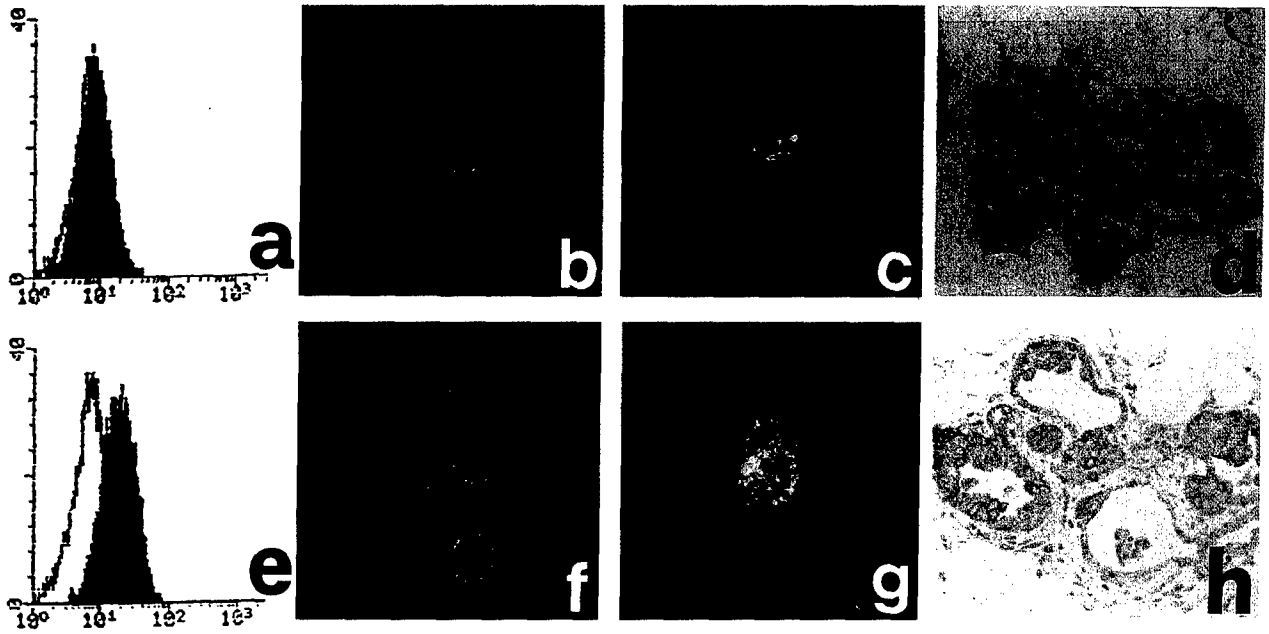

FIGURE 5. Down-modulation of cell membrane expression of r-p185. *a* and *e*, Flow cytometric analysis of the ability of sera to bind TUBO cells. Open profiles, Cells stained with secondary Ab; shaded profiles, cells stained with sera pool from BALB-neuT mice before (*a*) or after DNA vaccination (*e*). The ordinate represents the number of cells and the abscissa reports the fluorescence intensity in arbitrary logarithmic units. *b*, *c*, *f*, and *g*, Confocal analysis of r-p185 expression after a 3-h incubation of TUBO cells at 4°C (*b* and *f*) or at 37°C (*c* and *g*) with sera from BALB-neuT mice before (*b* and *c*) and after DNA vaccination (*f* and *g*). Immunohistochemistry reveals that the majority of epithelial cells in hyperplastic lesions from ECD-TM-immunized mice at 33 wk of age express the r-p185 only in the cytoplasm (*h*), whereas a clear membrane and cytoplasm r-p185 positivity are evident in similar lesions displayed much earlier (about at 6–10 wk of age) by control BALB-neuT mice (*d*). Original magnification: *d* and *h*, × 400.

the mammary glands is the production of anti-r-p185 Ab. Although proliferating TUBO cells expressing high amounts of r-p185 on their membrane are unable to elicit a detectable Ab response in both BALB/c and BALB-neuT mice, a significant titer of anti-r-p185 Ab was found in the sera from our immunized mice. It increased when four vaccinations were given instead of two, although BALB-neuT mice consistently produced lower Ab than BALB/c mice. Ab titer is not markedly affected by the challenge and rejection of TUBO cells nor by the development of mammary carcinomas.

Anti-r-p185 Ab induces a functional block of r-p185 receptor function (14), down-regulates its expression on the cell membrane (14, 40), impedes its ability to form the homo- or heterodimers that spontaneously transduce proliferative signals to the cells (40, 41), and blocks its ability to bind ligands (42), as has been observed with anti-Her-2/*neu* mAb. These mAb also significantly suppress the growth of transplantable $p185^+$ tumors (43, 44) and the onset of mammary carcinomas in Her-2/*neu*-transgenic mice (40), and delay tumor growth in patients with Her-2/*neu*-positive tumors (45). The morphological features of inhibited proliferation associated with marked membrane down-modulation of r-p185 and diminished nuclear positivity of PCNA characterizing the progression of both preneoplastic lesions and incipient carcinomas point to direct inhibitory activity on the part of anti-r-p185 Ab. A reduced r-p185 expression could be sufficient for the reversal of their transformed phenotype into a more normal one (14, 40). The high and homogeneous r-p185 membrane expression in advanced lobular carcinomas grown in ECD-TM-immunized and control BALB-neuT mice suggests that its down-regulation becomes less inducible as carcinogenesis progresses.

The success of DNA vaccination in the inhibition of $p185^+$ carcinomas appears to mostly depend on an Ab response to a growth factor receptor whose down-regulation slows the preneoplastic cell proliferation and tumor development. This inhibition mechanism is different from immunological destruction of the malignant cells. However, in immunized mice, leukocytes present at the tumor growth site may also play an important regulatory role (28, 33, 39). Moreover, TUBO cells are rejected by ECD-TM-immunized BALB/c mice that display a high titer of IgG2a, IgM, and IgG1 anti-r-p185 and a massive cellular infiltrate. These Ab isotypes activate PMN and other cells to mediate Ab-dependent cell-mediated cytotoxicity (44–48) and complement-dependent cytotoxicity (IgG2a and IgM), and inhibit the growth of the $p185^+$ tumor in vivo (44). In BALB-neuT mice, ECD-TM plasmid immunization elicits a much lower titer of IgG2a, IgG1, IgM, and IgA anti-r-p185. In these mice, only a partial resistance to a TUBO challenge but major impairment of the progression of carcinogenesis was found. In both BALB/c- and BALB-neuT-immunized mice, CTL do not appear to play a major role, whereas IgG, IgM, and IgA may synergistically promote Ab-dependent cell-mediated cytotoxicity (44, 47–49).

In BALB-neuT mice, our results also fail to show a direct correlation between the titer of anti-p185 Ab and protection from TUBO challenge and inhibition of Her-2/neu carcinogenesis. Several issues may make this correlation less linear such as, for instance, the epitopes recognized by anti-r-p185 Ab, the isotypes of the Ig-elicited and the Ab-dependent mechanisms that are mostly responsible for protection from tumor formation. To definitively address the role of anti-r-p185 Ab in the inhibition of Her-2/*neu* carcinogenesis, we are currently breeding BALB-neuT mice devoid of B cells functions (BALB-neuT/μMT) (50).

The present findings extend and corroborate in a much more aggressive model of carcinogenesis our earlier demonstration that DNA vaccination halts the slower and more limited Her-2/*neu* carcinogenesis taking place in FVB mice (19). Despite the similarity of mammary carcinogenesis in BALB-neuT mice and women (32), the mechanisms of tolerance to r-p185 could be different from those to self p185 in women. However, the r-p185 amino acid sequence is very similar to that of mouse p185, and ECD-TM DNA vaccination and in vitro restimulation never elicited a significant CTL response. Even if the data from BALB-neuT-transgenic mice cannot be directly translated to humans, they show that tolerance to an Ag already markedly expressed during the third wk of age can be partially broken. ECD-TM plasmid immunization appears to be more effective than other forms of anti-r-p185 vaccination (17, 18). Considering that Her-2/*neu* is overexpressed by a substantial proportion of human mammary carcinomas and that many women with a high risk of cancer are being recruited in ongoing epidemiological, genetic, molecular, and radiological screening programs, DNA vaccination could be envisaged as a new prospect in the prevention of carcinogenesis due to the overexpression of oncogenic growth factor receptors (51).

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SALIVARY CARCINOMA IN HER-2/neu TRANSGENIC MALE MICE: AN ANGIOGENIC SWITCH IS NOT REQUIRED FOR TUMOR ONSET AND PROGRESSION

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Morphologic examinations of salivary gland neoplasias arising in male BALB/c (H-2^d) mice carrying the activated HER-2/neu (BALB-NeuT) indicate that expression of the oncogene product in the ductal-acinar structures results in a very human-like acinic cell adenocarcinoma with a smoldering course and infrequent metastatization. Typical and then atypical hyperplasia of ducts and acini preceded the rise of salivary tumors that originated from the confluence of multiple ductal hyperplastic foci, while hyperplastic acini behaved as an abortive preneoplastic lesion. The vascular network in normal, hyperplastic and neoplastic salivary tissue was analysed to see whether activation of the angiogenic process is essential in salivary gland carcinogenesis. Immunostaining with anti-endothelial cells (anti-CD31), anti-β_3 integrin and anti-laminin antibodies revealed that microvessel density was significantly higher in normal and hyperplastic than in neoplastic tissue, in which no signs of new vessel sprouting were found. Assessment of angiogenic factor expression indicates a low presence of VEGF in normal, hyperplastic and neoplastic epithelium, while bFGF was preferentially produced but not exported by neoplastic cells and remained in a cell-associated form. Our data suggest that normal salivary gland vascularization is able to support tumor onset and development with no need for an angiogenic switch. *Int. J. Cancer* 88:329–335, 2000.
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The annual world incidence of salivary gland tumors is 0.4 to 0.5 cases per 100,000 persons (Auclair *et al.,* 1991), and they account for 2% to 6.5% of all head and neck neoplasms (Auclair *et al.,* 1991; Spiro, 1986). An overview of several large case series shows that between 74% and 80% of all primary epithelial tumors occur in the parotid glands, 7% to 11% in the submandibular glands, and less than 1% in the sublingual glands (Auclair *et al.,* 1991; McKenna, 1984; Eveson and Cawson, 1985). The overall incidence peaks in the 6th and 7th decades of life, though the incidence of specific histotypes, such as mucoepidermoid carcinomas and acinic cell adenocarcinomas, peaks in the 3rd and 4th decades (Ellis and Auclair, 1996).

In the salivary gland, as in the breast, ovary and uterus, amplification and over-expression of the HER-2/neu oncogene may be carcinogenic (Slamon *et al.,* 1989; Hetzel *et al.,* 1992; Press *et al.,* 1994) and immunohistochemical detection of HER-2/neu expression usually implies a worse prognosis.

Generation of a mouse strain transgenic for the activated rat HER-2/neu oncogene (Di Fiore *et al.,* 1990; Lucchini *et al.,* 1992; Boggio *et al.,* 1998) has permitted investigation of a spontaneously arising mouse mammary carcinoma and evaluation of the *in vivo* role of HER-2/neu in its cancerogenesis. It was observed that activation of angiogenesis during prior mammary hyperplasia preceding cancer was a critical step in the progression to tumor (Di Carlo *et al.,* 1999). However, the results of several studies suggest that tumors do not always require angiogenesis induction, particularly when they arise within a richly vascularized tissue from which existing blood vessels may be coopted (Pezzella *et al.,* 1997).

This article describes the salivary gland tumor spontaneously arising in male mice of the same transgenic strain. The possibility that an angiogenic switch is an essential step, even in carcinogenesis involving richly vascularized organs such as salivary glands, is also investigated.

MATERIAL AND METHODS

Mice

A transgenic CD1 random-bred breeder male mouse (no. 1,033) carrying the mutated rat HER-2/neu oncogene driven by the MMTV promoter (Tg-NeuT, provided by Dr. L. Clerici, Euratom, Ispra, Italy) (Lucchini *et al.,* 1992) was mated with BALB/c females (H-2^d; Charles River, Calco, Italy). The progeny was screened for the transgene by PCR. Transgene-carrying males were backcrossed with BALB/c female for more than 12 generations, and HER-2/neu BALB/c mice (BALB-NeuT) were used in these experiments. Males of BALB-NeuT transgenic line show an MMTV-driven over-expression of the transgene in the salivary gland and a definite tumor growth involving the salivary gland epithelium (Lucchini *et al.,* 1992). Individually tagged males were used in our study. Starting at the age of 4 weeks, their salivary glands were inspected once a week, and masses were measured with calipers in the 2 perpendicular diameters. Progressively growing masses >3 mm mean diameter were regarded as tumors. Mice were killed at week 40 when these masses exceeded 2 cm mean diameter.

Morphologic analysis

Groups of 2 mice were killed at week 4 and week 5 and then every 2 or 3 weeks until week 40. For histologic evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin, PAS and PAS-diastase. For electron microscopy, specimens were fixed in cacodylate-buffered 2.5% glutaraldehyde, postfixed in osmium tetroxide and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate-lead citrate. For immunohistochemistry, formalin-fixed, paraffin-embedded or acetone-fixed cryostat sections were incubated for 30 min with anti-endothelial cells (mEC-12.324, provided by Dr. A. Vecchi, Istituto M. Negri, Milano, Italy), anti-CD61 (integrin β_3 chain) (PharMingen, San Diego, CA), anti-collagen Type IV (Chemicon, Temecula, CA), anti-laminin (Becton Dickinson, Bedford, MA), anti-vascular endothelial growth factor (VEGF), anti-basic fibroblastic growth factor (bFGF), anti-neu (C-18; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-proliferating cell nuclear antigen (PCNA; Ylem, Roma, Italy) antibodies. After washing, the sections were

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overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Igs (Vector Labs, Burlingame, CA) for 30 min. Unbound Ig was removed by washing, and the slides were incubated with avidin-biotin complex (ABC)/alkaline phosphatase (AP) (Dako, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by 3 pathologists in a blind fashion. From mice with multiple hyperplastic foci or tumors, 2 or more samples (1/tumor growth area) and 10 randomly chosen fields in each sample were evaluated for each point determination. The expression of angiogenic factors was defined as absent (−) or as scarcely (±), moderately (+) or frequently (++) present on cryostat sections tested with the corresponding antibody. For microvessel counts, individual microvessels were counted under a microscope × 400 field (× 40 objective and × 10 ocular lens, 0.180 mm^2 per field). The rate of immunoreactivity for PCNA was obtained by counting the number of positive cells/number of total cells in the tubular and acinic structures under a microscope × 600 field (× 60 objective and × 10 ocular lens, 0.120 mm^2 per field).

In situ *detection of apoptosis*

Sections were dewaxed, treated with proteinase K and incubated with 0.5% hydrogen peroxide in methanol for 1 hr.

The specimens were then exposed for 1 hr at 37°C in a moist chamber to the TUNEL labelling mix containing 0.3 U/μl calf thymus terminal deoxynucleotidyl transferase (TdT), 5 to 8 μM bio-UTP in TdT buffer (Roche Molecular Biochemicals, Monza, Italy) in distilled water. Following washing (4 PBS baths of 5 min each at room temperature), the specimens were resaturated (30 min) in 3% BSA and 20% normal sheep serum in 1% w/v blocking reagent (Roche Molecular Biochemicals) in 0.1 M tris-buffered saline (TBS). They were then treated for 30 min at RT with 1:100 dilution of streptavidin/horseradish peroxidase, followed by washing and 0.05% 3-3′-diaminobenzidine-tetrahydrochloride (DAB) color reaction (Dako, Glostrup, Denmark). To provide a quantitative analysis, we determined the apoptotic index for tumor cells. This represents the number of TUNEL-positive tumor cells among 100 tumor cells. We counted the number of target cells in at least 10 high-power fields. Areas of necrosis were excluded since they comprised some pseudo-positive cells.

mRNA for angiogenic factors

Total RNA was prepared from BALB/c normal salivary gland tissue, from BALB-NeuT neoplastic lesions and from primary cultured cells by using Ultraspec (Biotex Lab., Houston, TX). Two micrograms of RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (200 U) in 50 μl of reaction mixture with oligo dT and dNTP (GIBCO BRL, Paisley, UK). The cDNA were tested for the presence of murine glucose-3-phosphate-dehydrogenase (G3PDH), VEGF and bFGF sequences in PCR reactions (Gene Amp Kit; Perkin Elmer Cetus, Nowalk, CT) performed in 20 μl volumes and amplified by 35 PCR cycles with specific primer pairs prepared by us (VEGF) or from Stratagene (La Jolla, CA) (G3PDH, bFGF).

Cell lines

SM-1 cells were established from a tumor that arose in a parotid gland of a 29-week-old male BALB-NeuT mouse. Its histological appearance was that of an acinic cell carcinoma. Tissue from this tumor was minced and adapted to grow in RPMI 1640 (GIBCO) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FCS (GIBCO) at 37°C in a humidified 5% CO_2 atmosphere. SM-1 cells are positive for neu, VEGF and bFGF (immunostaining). All the assays were performed before the 5th passage.

Preparation of conditioned media and cell extracts

Cells were cultured in 100 mm plastic Petri dishes, washed with PBS and incubated with 10 ml of fresh RPMI 1640 (supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2% heat-inactivated FCS), which was collected after an additional 3-day incubation. After low-speed centrifugation (1,000 rpm, 10 min) to remove cellular debris, the conditioned medium (CM) was concentrated by ultrafiltration (Centriprep-10; Amicon, Milano, Italy). The sample was adjusted with PBS to 1 mg/ml of total protein.

Cell extract was prepared from monolayer cultures. After CM was removed from plastic dishes, cells were washed with PBS and harvested with 0.25% trypsin. Cell clones were then washed with PBS, resuspended in 1.0 ml of NP40 lysis buffer (150 mM Nacl, 10% NP-40, 50 mM Tris, pH 8.0) and incubated on ice for 30 min. The lysate was then spun at 10,000 g for 10 min.

Enzyme immunoassay for quantitative determination of bFGF

bFGF contained in CM and cell extracts was measured by a commercially available microtiter-based sandwich enzyme immunoassay system (Quantikine FGF basic immunoassay kit; R&D Systems, Minneapolis, MN). In brief, 200 μl of CM or cell extract containing 25 μg of total protein were added to each microtiter plate coated with a mouse monoclonal antibody (MAb) specific for bFGF and then incubated for 2 hr at RT. Following a wash, 200 μl of horseradish peroxidase-linked rabbit polyclonal antibody against bFGF were added and incubated for 2 hr at RT. Then, 200 μl of substrate solution (tetramethylbenzidine and hydrogen peroxide) were added and incubated for 20 min. After washing, 50 μl of stop solution were added. Optimal absorbance was read at 450 nm using a Dynatech spectrophotometer.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were analyzed for statistical significance using the χ^2 test and Student *t*-test, with $p<0.05$ as the significance cutoff.

RESULTS

Histologic and ultrastructural features

No BALB-NeuT male mice displayed palpable salivary gland lesions until 23 weeks of age when development of a tumor began that involved the parotid glands, first on one side and then on the other, and later (34 to 40 weeks) the submandibular glands, with infrequent lung metastases.

At 4 weeks of age, histological examination of parotid and submandibular glands revealed multiple foci of typical hyperplasia involving intercalated ducts and serous acini, rapidly followed by atypical hyperplasia at 6 weeks (Fig. 1). Duct hyperplasia was marked by an assembly of ductal structures organized in a nodular fashion, lined by a single layer of epithelial cells with a greater variability in size than in normal glandular tissue. Acinar hyperplasia was characterized by enlarged acini packed by polygonal cells with granular, slightly basophilic cytoplasm and hyperchromatic, frequently disproportionately large nuclei. The basal layer of flattened cells, morphologically identifiable as myoepithelial cells, was moderately reduced in the hyperplastic ducts and markedly in the enlarged acini.

Starting at the 13th week, the hyperplastic areas expanded to form by about the 20th week multiple small masses that combined in a larger tumor (Fig. 1*c*) consisting of large, round cells to polygonal, closely apposed cells forming sheets, nodules or aggregates. The predominant cell type displayed amphophilic to eosinophilic cytoplasm and round, basophilic to vesicular nuclei. These cells were smaller but similar in shape to many acinar cells and unreactive with PAS stain. A more evident acinar differentiation characterized by cells with granular PAS-positive cytoplasm and uniform, round, eccentric nuclei was often present.

Ultrastructural examination of duct hyperplasia revealed that it was due to an increase of ductal epithelial cells characterized by a pale-staining, organelle-poor cytoplasm that often contained a few secretory granules. Acinar hyperplasia was determined by the aggregation of large polygonal and usually binucleate or bilobate cells. The characteristic feature of their cytoplasm was the constant

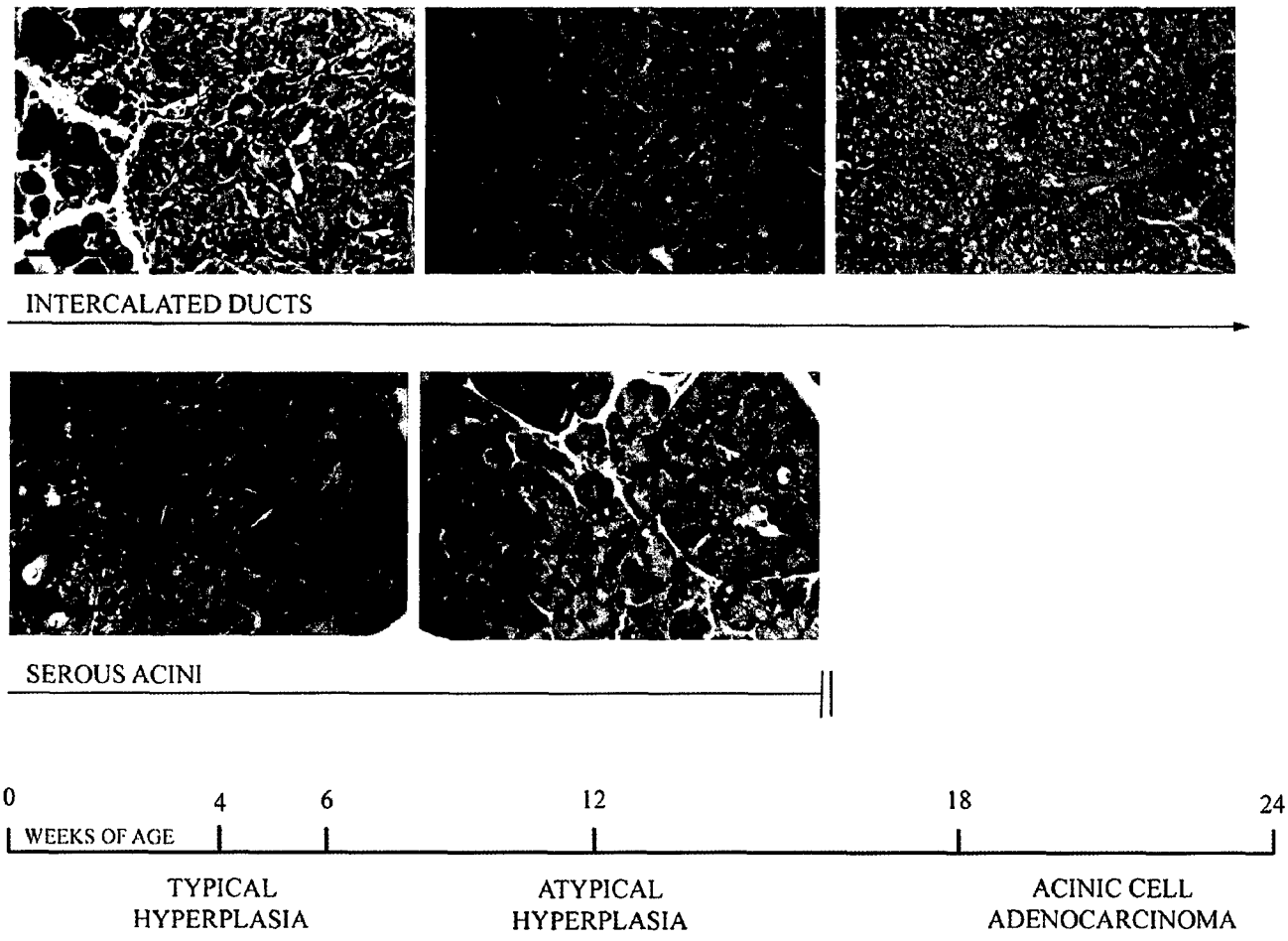

FIGURE 1 – Salivary tumor development in rat HER-2/neu transgenic male mice. Parotid gland of 4-week-old BALB-NeuT mice reveals multiple foci of typical hyperplasia involving intercalated ducts (*a*) and serous acini (*d*). Atypical hyperplasia of ducts (*b*) and acini (*e*) already evident at 6 weeks of age progressively grows, giving rise to a multifocal acinic cell adenocarcinoma in 19-week-old mice (*c*). Scale bar = 100 μm; × 400×.

presence of multiple, round, electron-dense secretory granules in well-developed tumor masses, though with marked differences in number, size and electron density, indicating that the tumors were adenocarcinomas of acinic cell type (Fig. 2*a,c*). The basal layer of flattened myoepithelial cells still present in hyperplasia was markedly rarefied or absent in adenocarcinoma.

The SM-1 cell line derived from the parotid gland tumor consisted of acinic cells with a fragile granular cytoplasm and dark nuclei (Fig. 4*a*). The granules were purple-red on periodic acid-Schiff (PAS) staining.

Histologic and ultrastructural features of these salivary gland carcinomas were identical to those of human acinic cell adenocarcinomas composed of nonspecific glandular cells (Ellis and Auclair, 1996).

Immunohistochemical analysis and apoptotic cell detection

Immunohistochemistry with anti-neu antibody performed at 3 weeks of age revealed that epithelial cells of normal gland were negative, except for some of those lining intralobular ducts close to sites of acinar differentiation. Later on, neu positivity was detectable without appreciable differences in ductal and acinar hyperplasia as in carcinomatous tissue (Fig. 3*a–c*).

Proliferating cell nuclear antigen (PCNA) expressed by a few epithelial cells in normal gland was moderately present in hyperplastic intercalated duct cells and slightly present in hyperplastic acinar cells. Its expression was high in carcinoma (Fig. 3*d–f* and Table I).

TUNEL analysis revealed that the percentage of apoptotic cells in carcinomatous tissue was slightly but not significantly higher than in hyperplastic and normal tissue (Fig. 4*b* and Table I).

Microvessel count indicated that the vascularization of hyperplastic foci was similar to that of normal gland, whereas in carcinoma it consisted of fewer but larger vessels (Fig. 4*g* and Table I).

Anti-β_3 chain staining showed that the adhesion receptor $\alpha_v\beta_3$ selectively expressed on growing vessels (Brooks *et al.*, 1994; Shattil, 1995) was almost absent in normal, hyperplastic and neoplastic salivary gland tissue (data not shown).

Immunohistochemical staining for angiogenic factors revealed that VEGF was scarcely expressed by epithelial cells in normal, hyperplastic and neoplastic tissue. Basic FGF (scarcely present in normal and hyperplastic salivary tissue) was clearly expressed in several (<50%) neoplastic epithelial cells but undetectable in the laminin and collagen type IV-rich delicate stroma surrounding the neoplastic sheets (Fig. 4*d* and Table I). Basic FGF and VEGF were immunohistochemically detected in cultured epithelial cells from salivary carcinomatous tissue.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and enzyme immunoassay detection of angiogenic factors

When the enzyme immunoassay for bFGF was performed on CM and cell extract from SM-1 neoplastic cells, the values de-

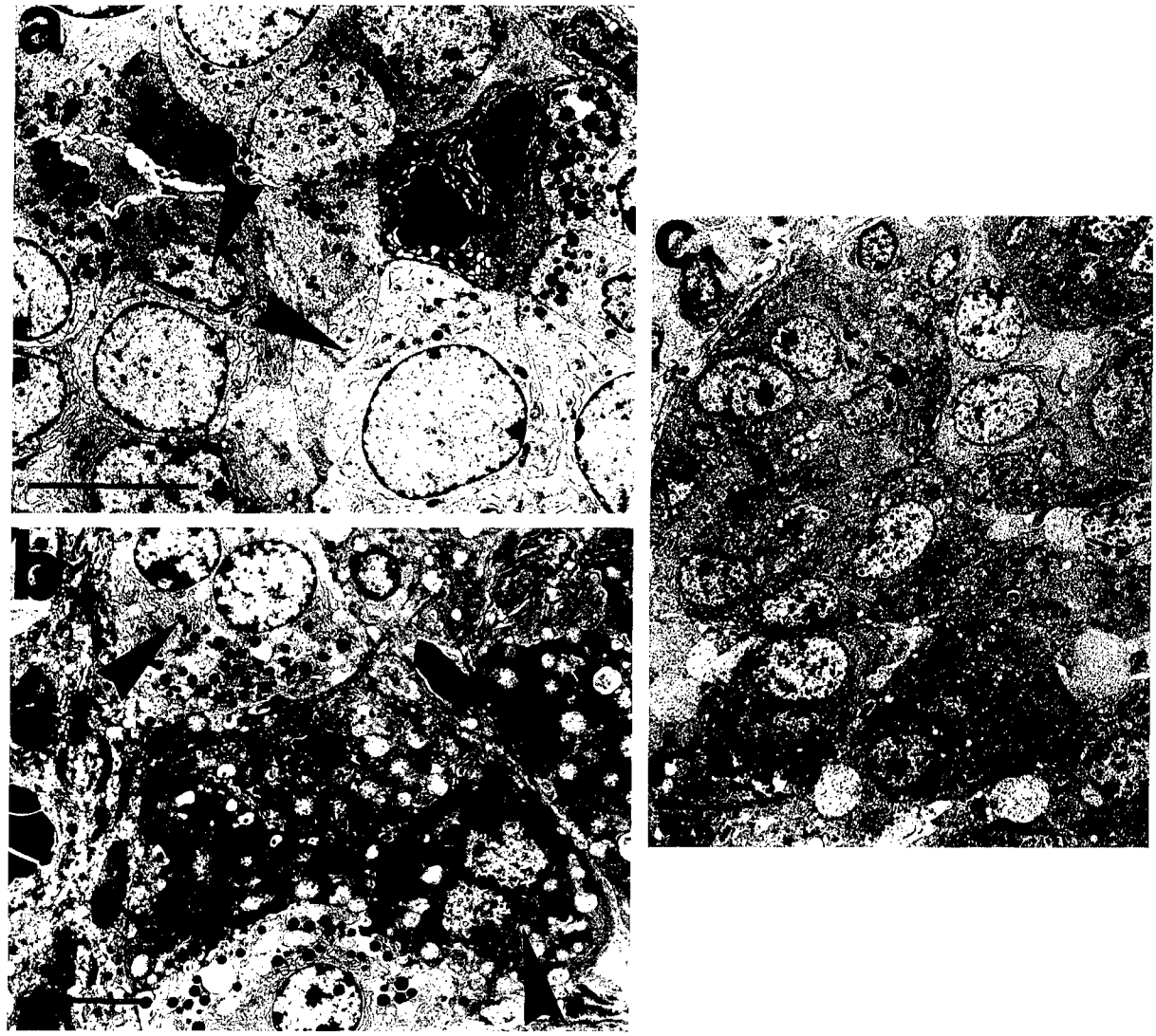

FIGURE 2 – Ultrastructural features of ductal (*a*) and acinar (*b*) atypical hyperplasia and adenocarcinoma arising in parotid gland of 8-week-old (*a,b*) and 22-week-old (*c*) BALB-NeuT mice, respectively. Ductal hyperplasia (*a*) consists of an increase of ductal epithelial cells with a pale-staining, organelle-poor cytoplasm containing a few secretory granules (arrowheads). Acinar hyperplasia (*b*) is formed of large polygonal cells with 2 or bilobate nuclei (arrowheads) and containing the typical cytoplasmic electron-dense secretory granules. Salivary adenocarcinoma (*c*) is formed of round to polygonal cells with a large round or oval nucleus and cytoplasmic electron-dense secretory granules that confirm its acinic cell origin. Scale bar = 10 μm; *a* × 2,750, *b* × 1900, *c* × 1450×.

tected were <0.5 for CM and 21.3 ± 0.18 pg bFGF/μg protein for cell extract.

The mRNA of bFGF (Fig. 4*e*) and VEGF was evidenced by RT-PCR in normal and neoplastic tissue and in the SM-1 neoplastic cells.

DISCUSSION

Expression of the activated HER-2/neu oncogene in male BALB-NeuT mice results in a slow-growing, multifocal acinic cell adenocarcinoma that involves first the parotid and then the submandibular gland. Preneoplastic lesions preceding carcinoma had the features of typical and then atypical hyperplasia of intercalated ducts and acini. Diagnosis of acinic cell adenocarcinoma was based on the presence of sheets, nodules or aggregates of large, round to polygonal cells containing multiple electron-dense secretory granules, which increased in number until they stuffed all the cytoplasm in tumor areas with more pronounced acinar differentiation (Ellis and Auclair, 1996). A delicate stroma was interposed among the neoplastic cell aggregates.

Detection of neu positivity in epithelial cells of ductal and acinar hyperplasia and in neoplastic cells of acinic cell adenocarcinoma clearly suggests that the expression of neu is the hallmark of neoplastic transformation and leads to epithelial cell proliferation, as confirmed by the elevated nuclear positivity for PCNA in the neu positive areas. The cell proliferation rate was higher in the ductal than in the acinic hyperplasia (Martinez-Madrigal and Micheau, 1992), though low compared to a fully developed acinic cell carcinoma, while no appreciable differences in the expression of neu were detectable. Histologic examination revealed that foci of acinic hyperplasia did not expand and form a tumor mass. The tumor mass, in fact, was generated by the confluence of multiple foci of ductal hyperplasia.

A possible explanation of this peculiar tumorigenesis is that the ductal walls are formed of a high proportion of relatively undifferentiated cells (to be considered as reserve cells), whereas the

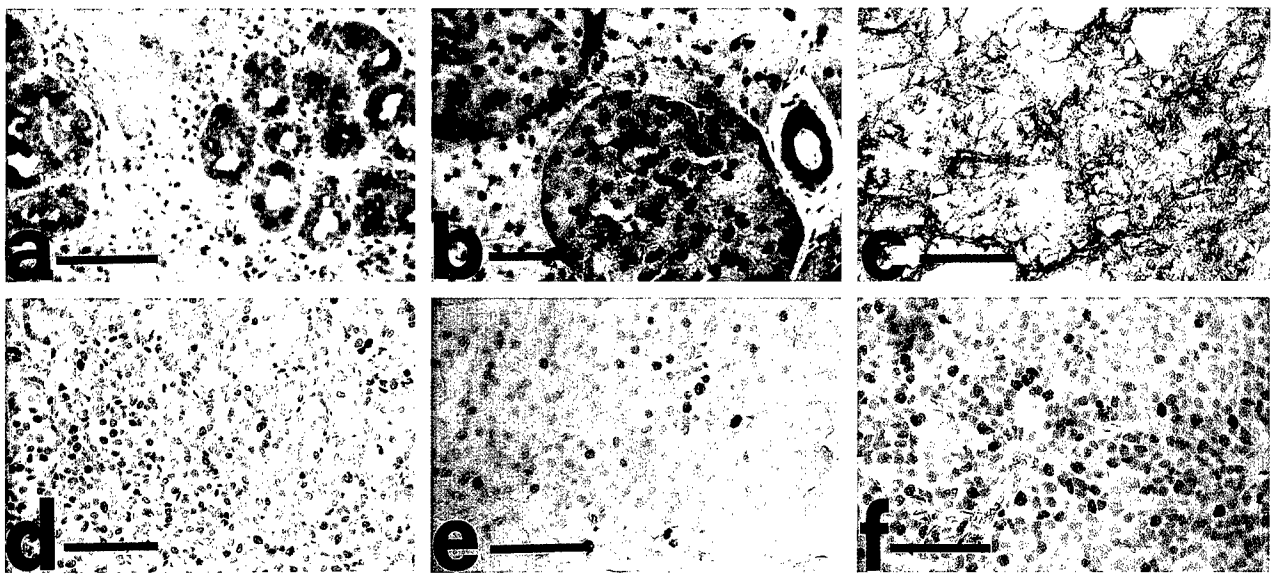

FIGURE 3 – Immunohistochemical analysis of neu and PCNA expression in hyperplastic (*a,b,d,e*) and carcinomatous (*c,f*) tissues of 8-week-old and 22-week-old BALB-NeuT mice, respectively. Cryostat sections tested with anti-neu (C-18) antibody (*a–c*) showing the expression of neu oncogene product in ductal hyperplasia (*a*), acinic hyperplasia (*b*) and acinic cell adenocarcinomas (*c*). Formalin-fixed, paraffin-embedded tissue sections tested with anti-PCNA antibody (*d–f*) showing that the cell proliferation rate is moderate in hyperplastic intercalated ducts (*d*), low in hyperplastic acini (*e*) and high in acinic cell adenocarcinomas (*f*). Scale bar = 100 μm; *a–e* × 400×.

TABLE I – ANALYSIS OF PROLIFERATION RATE, APOPTOTIC CELLS, MICROVESSELS AND ANGIOGENIC FACTORS IN NORMAL, HYPERPLASTIC AND NEOPLASTIC SALIVARY GLANDS OF BALB-NeuT MALE MICE

	Normal gland	Hyperplastic foci (8 weeks)		Adenocarcinoma (22 weeks)
		Intercalated ducts	Serous acini	
Epithelial cells:				
PCNA	1.3% ± 0.9%[1]	13.3% ± 2.5%*	5.7% ± 2.1%*	21.1% ± 4.3%*·**
Apoptotic cells (TUNEL)	0.4% ± 0.2%	0.6% ± 0.2%	0.4% ± 0.3%	0.9% ± 0.4%
Microvessels	63.2 ± 8.1	65.4 ± 7.4	57.6 ± 9.3	15.6 ± 4.9*·**
Angiogenic factors:				
VEGF	±	±	±	±
bFGF	±	±	±	+[2]

[1]Quantitative studies were performed as described in Material and Methods.–[2]bFGF was clearly expressed in <50% of neoplastic cells but undetectable in the stroma.–*·**Values significantly ($p < 0.01$) different from those of normal gland* and hyperplastic foci**.

acinic structures are formed of highly specialized secretory cells with a low self-renewing rate (Martinez-Madrigal and Micheau, 1992).

As in carcinoma of the breast, ovary and endometrium (Slamon *et al.*, 1989; Hetzel *et al.*, 1992), amplification and over-expression of Her-2/neu oncogene occur in a distinct percentage of primary human salivary gland neoplasms (Press *et al.*, 1994; Karja *et al.*, 1994). Since the long natural history of human salivary tumors, the advanced age of many patients (Ellis and Auclair, 1996) and the few cases collected in most series are all limitations on their investigation, a murine model would be useful for this purpose and in the search for ways to block the carcinogenic steps.

In our transgenic model, salivary gland tumors develop with a smoldering behavior as in humans. We therefore investigated the involvement of tumoral angiogenesis in this slow growth. The immunohistochemical results obtained with an anti-endothelial cell antibody (anti-CD31) showed that the microvessel density of salivary acinic tumor is low compared with the vascularization of the surrounding normal tissue. Even so, signs of ischemic necrosis were almost absent inside the tumor, suggesting that the blood supply was sufficient for this indolent growth.

It has been reported that a distinct subset of tumors grows initially by co-opting host vessels (Holash *et al.*, 1999; Holash *et al.*, 1999) which first regress and then expand as a hypervascular plexus at the tumor border. In our setting, no signs of significantly necrotic or angiogenic events were evident, suggesting that the growth kinetics was slow, but progressive as a result of tumor cell adaptability. It is likely that salivary tumor cells elude their defective angiogenic factor synthesis or release by co-opting vessels from the richly vascularized normal tissue.

The low vessel density we found inside the tumor mass is a reasonable explanation for the lack of biological aggressiveness and the low metastatic capability in this murine model, as in many human salivary gland tumors.

The lack of effective tumor angiogenesis confirmed by the almost absent integrin β_3 expression in endothelial cells and the absence of an aggressive tumor behaviour are presumably related to the permanent cell-associated state of bFGF (Kandel *et al.*, 1991) and the steady level of VEGF expression in normal as in neu-expressing hyperplastic and neoplastic salivary tissue. The lack of its extracellular release may hamper efficient angiogenesis.

Acquisition of a metastatizing phenotype has been associated with an increase in bFGF release in human salivary gland cancer cells (Azuma *et al.*, 1997). Furthermore, the switch from a stored to an exported form of bFGF in aggressive fibromatosis and fibrosarcoma cells correlates not only with histologic neovascular-

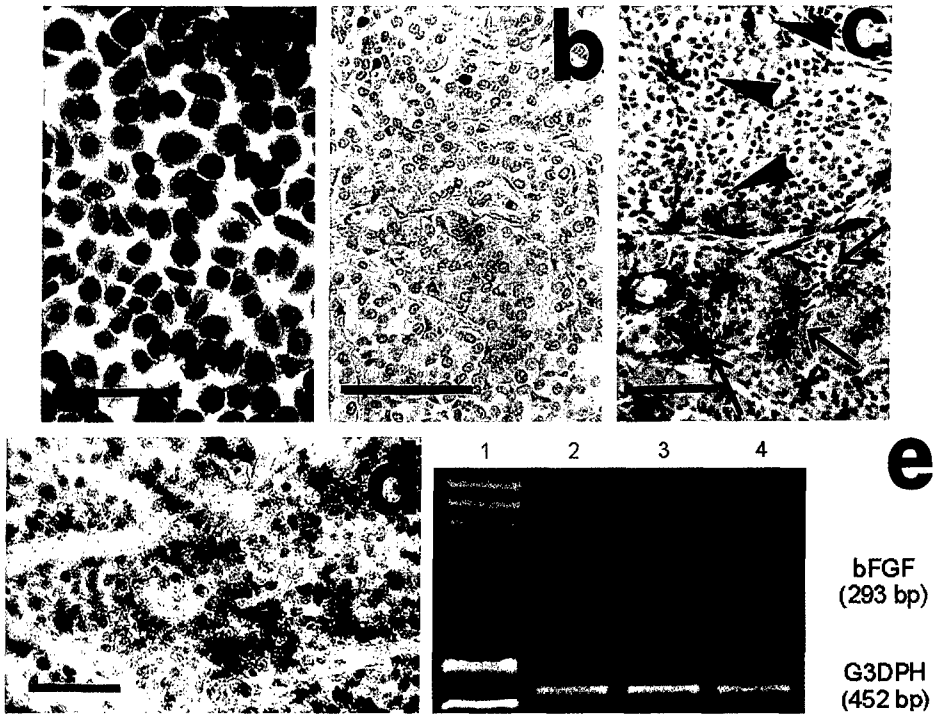

FIGURE 4 – Morphologic feature of a salivary tumor-derived cell line and detection of apoptotic cells (*b*), microvessel network (*c*), bFGF protein (*d*) and mRNA (*e*) expression in 22-week-old BALB/NeuT salivary tumors. (*a*) PAS staining of a cultured SM-1 cell line derived from the parotid gland tumor shows epithelial acinic cells with a fragile granular cytoplasm and dark nuclei. (*b*) TUNEL assay with formalin-fixed, paraffin-embedded tissue section reveals that a very small percentage of salivary tumor cells undergoes apoptosis. (*c*) Cryostat section tested with anti-CD31 antibody shows a rich vascularization in normal gland (arrows), while few blood vessels are present in carcinoma (arrowheads). (*d*)The cryostat section tested with anti-bFGF antibody shows that bFGF is confined to the cytoplasm of several tumor cells and not detectable in the surrounding stroma. Scale bar = 100 μm; *a,b* × 630; *c,d* × 400×. (*e*) mRNA specific for mouse bFGF in normal (lane 2), neoplastic salivary gland tissue(lane 3) and salivary cancer cell line (lane 4) determined by RT-PCR. Lane 1 contains the size marker φX174DNA/Hae III.

ization but also with the tumorigenicity of the cells themselves (Kandel *et al.,* 1991).

The expression of neu in HER-2/neu transgenic mice mainly results in mammary tumors in female (Boggio *et al.,* 1998) and salivary tumors in male animals (Lucchini *et al.,* 1992). In the mammary hyperplasia that precedes a tumor, angiogenesis activation by bFGF, VEGF, etc., gives rise to a vascular network capable of supplying all the subsequent tumor growth (Di Carlo *et al.,* 1999). In normal salivary gland tissue, blood vessels are well represented and widely distributed. This vascular support may represent a good soil for tumor onset and development with no need for an angiogenic switch.

In conclusion, our data suggest that an angiogenic switch may not be an essential step for the development of slow-growing human tumors, particularly those arising in well-vascularized organs.

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p185^{neu} PROTEIN IS REQUIRED FOR TUMOR AND ANCHORAGE-INDEPENDENT GROWTH, NOT FOR CELL PROLIFERATION OF TRANSGENIC MAMMARY CARCINOMA

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Transgenic FVB-NeuN mice (N202) bearing the rat *neu* protooncogene driven by the mouse mammary tumor virus promoter/enhancer develop focal mammary carcinomas overexpressing the *neu*-encoded p185^{neu} protein. *In vitro* expression of p185^{neu} among mammary carcinoma cultures was heterogeneous, and we could establish some cell lines and clones displaying a complete loss of p185^{neu} expression, along with others with very high p185^{neu} protein level. Upon *in vivo* injection, p185^{neu}-positive cells gave rise to fast-growing tumors with a short latency, while p185^{neu}-negative cells required a very long latency time, and the resulting tumors were invariably p185^{neu}-positive. The lower growth ability of p185^{neu}-negative cells *in vivo* was also confirmed in athymic nude mice. *In vitro,* analysis of anchorage-independent growth in soft agar revealed colony formation from p185^{neu}-positive but not p185^{neu}-negative cells. The direct involvement of p185^{neu} in clonogenicity was demonstrated by the inhibition of p185^{neu}-positive colony growth in soft agar in the presence of an anti-p185^{neu} monoclonal antibody. By contrast, a higher level of anchorage-dependent clonogenic growth and proliferation was observed in p185^{neu}-negative cells as compared to p185^{neu}-positive cells, thus explaining the relative ease with which p185^{neu}-negative cell lines and clones were established *in vitro*. Together, the results indicate that p185^{neu} expression can lead to tumor formation and metastasis through the modification of intrinsic properties of cells related to anchorage-independent growth ability rather than to proliferation or host-dependent mechanisms.
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The rat *neu* protooncogene and its human homologue *HER-2* encode a 185 kDa transmembrane glycoprotein (p185$^{HER-2/neu}$) with intrinsic tyrosine kinase activity related to the epidermal growth factor receptor (King *et al.,* 1985; Coussens *et al.,* 1985). Amplification and overexpression of *HER-2* has been implicated in the pathogenesis of several human malignancies, including breast, ovarian and lung carcinomas (Slamon *et al.,* 1989; Kern *et al.,* 1994; Felip *et al.,* 1995), and its overexpression correlates significantly with poor prognosis in subsets of patients with breast cancer (Slamon *et al.,* 1987; Rilke *et al.,* 1991; Pupa *et al.,* 1996). *In vitro* studies using rodent cells indicated that p185^{neu} overexpression *per se* is sufficient to induce malignant transformation (Di Fiore *et al.,* 1987). In several strains of transgenic mice carrying the activated rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer, early onset of transgene expression in the mammary epithelium of female and male mice results in the synchronous appearance of tumors involving all mammary glands (Muller *et al.,* 1988). FVB-NeuN mice (line N202) overexpressing the unactivated *neu* transgene driven by the MMTV promoter/enhancer develop focal mammary tumors, but only in females and with a long latency (Guy *et al.,* 1992). Together, these findings identify *HER-2/neu* as a potent oncogene. However, there is no direct evidence to indicate a causal relationship between p185^{HER-2} overexpression and malignancy, and the prognostic value of p185^{HER-2} overexpression *per se* is still controversial (Toikkanen *et al.,* 1992; Seshadri *et al.,* 1993; Hartmann *et al.,* 1994; Schonborn *et al.,* 1994; Revillion *et al.,* 1998). Clinical and molecular data indicate a much lower incidence of *HER-2* gene amplifications in distant metastases than in primary tumors (Driouch *et al.,* 1997), and the risk of metastasis is actually higher in breast cancer patients with p185^{HER-2} underexpressing primary tumor than in patients with normal or overexpressed p185^{HER-2} protein (Koscielny *et al.,* 1998).

The role of *HER-2/neu* gene in tumor transformation and progression is still unclear. Some experimental data suggest that *HER-2/neu* acts on proliferation since the signaling pathway of p185$^{HER-2/neu}$ involves MAP kinase activation; indeed, a very strong association between p185^{HER-2} overexpression and high number of mitoses has been reported in human breast carcinomas (Rilke *et al.,* 1991). However, other data suggest a role for *HER-2* gene in metastatic potential or in induction of hormone independence in a manner unrelated to cell proliferation (Yu *et al.,* 1994; Tan *et al.,* 1997). Furthermore, the dual role of *HER-2* activation in proliferation or differentiation, depending on the cell type has been demonstrated either by transfection of the *HER-2* gene or by treatment of cells with anti-p185^{HER-2} antibodies (Bacus *et al.,* 1992; Peles *et al.,* 1993; Giani *et al.,* 1998).

To investigate the role of *HER-2/neu* overexpression in tumorigenesis, we used the FVB-NeuN mouse strain (N202), which is transgenic for the rat *neu* protooncogene (Guy *et al.,* 1992) and represents a faithful model of human tumors overexpressing the p185^{HER-2} oncoprotein. We found that a complete loss of p185^{neu} oncoprotein expression is not uncommon among cells derived from transgenic mammary carcinomas, and we took advantage of this model to investigate the properties of interconverting p185^{neu}-positive and p185^{neu}-negative carcinoma cells. Our results indicate that the p185^{neu} oncoprotein does not contribute to the unrestricted proliferation of mammary carcinoma cells but is indispensable for the clonogenic anchorage-independent growth that underlies the ability to form progressive tumors *in vivo*.

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Appendix 6

MATERIAL AND METHODS

Transgenic mice and spontaneous mammary carcinomas

A colony of FVB-neuN mice, transgenic line N202 (Guy *et al.,* 1992; Boggio *et al.,* 1998), was established in our animal facilities from breeding pairs obtained from Dr. W.J. Muller, McMaster University, Hamilton, Ontario, Canada. Transgenic line N202 was derived by microinjection into the pronuclei of FVB/N fertilized one-cell zygotes of the *SphI-EcoRI* fragment excised from the plasmid pMMTVneuN, containing the *HindIII-EcoRI* fragment encoding the unactivated rat neu cDNA and SV40 polyadenylation and splicing signals from pSV2neuN (Guy *et al.,* 1992). Both virgin and breeder females of this transgenic line develop spontaneous mammary carcinomas that give rise to distant lung metastases (Guy *et al.,* 1992; Boggio *et al.,* 1998). Mice were maintained under strict inbreeding conditions. The presence of the rat *neu* transgene was routinely checked by polymerase chain reaction (PCR) on tail DNA using primers hybridizing to vector (5′-ATCGGTGATGTCGGCGATAT-3′) and to MMTV sequences (5′-GTAACACAGGCAGATGTAGG-3′). Female mice developed mammary carcinomas with a mean latency time of about 40 weeks. Tumors were either dissociated enzymatically for cytometric analysis of p185^{neu} expression or processed for morphologic analysis (Lollini *et al.,* 1998).

Morphologic analysis

For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin-eosin. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with a goat polyclonal IgG recognizing rat p185^{neu} (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), washed and overlaid with biotinylated anti-goat IgG (Vector, Burlingame, CA) for 30 min. After washing to remove unbound Ig, slides were incubated with avidin biotin complex/alkaline phosphatases (Dako, Glostrup, Denmark).

Enzymatic dissociation of tumors

Tumor samples were freed from hemorrhagic and necrotic parts, washed in phosphate-buffered saline (PBS), finely minced with scissors and digested with a standard tissue culture grade trypsin-EDTA solution (0.5 mg/ml trypsin, 0.2 mg/ml EDTA, Life Technologies, Milan, Italy) at 37°C for 15 min; dissociated cells were washed twice in PBS and counted in a hemocytometer. Previous tests using cell cultures showed that this enzymatic treatment does not affect neu antigens.

Establishment of transgenic mammary carcinoma cell cultures

A series of cell lines and clones was established at Istituto di Cancerologia from transgenic mammary carcinomas. Tumor samples minced with scissors were seeded in tissue culture flasks in Dulbecco's modified minimal essential medium (DMEM) + 20% fetal bovine serum (FBS) (Life Technologies) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cultures were periodically washed briefly (1–2 min) with trypsin-EDTA to detach contaminating fibroblasts without damage to epithelial areas. When the epithelial monolayer reached confluency, usually 2–5 months after plating, cells were subcultured at low split ratios (usually 1:2). Established cell lines and clonal derivatives were routinely subcultured twice weekly at 1:4–1:8 split ratios.

Flow cytometry

The product of the transgene, rat *neu,* was detected using monoclonal antibody 7.16.4 (Oncogene Research Products, Cambridge, MA). Cells were stained in a standard indirect immunofluorescence procedure (De Giovanni *et al.,* 1991) with primary antibody followed by a fluorescein-conjugated anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). After final washings, cells were resuspended in PBS containing 1 μg/ml of ethidium bromide to gate out dead cells, and analyzed by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Tumor samples were analyzed after gating for cell dimension (forward scatter) and granularity (side scatter) to exclude debris, passenger leukocytes and erythrocytes.

Tumorigenicity and metastasis studies

Healthy young (8–16 weeks old) female transgenic mice or 5-week-old *nu/nu* female mice on Swiss CD-1 background (Charles River Laboratories, Calco, Italy) were used for the analysis of tumorigenicity and metastatic ability of cultured cells. Tumors were induced by injecting mice subcutaneously (s.c.) in the right inguinal region with 0.2 ml of a single-cell suspension containing 10^6 viable cells. Tumor incidence and growth were evaluated twice weekly. Neoplastic masses were measured with calipers; tumor volume was calculated as $\pi/6\,[\sqrt{(a\,b)}]^3$ in which a and b are two perpendicular major diameters. Experimental metastases were evaluated 30 days after the injection of 10^5 cells in a lateral tail vein. Lung nodules were contrasted with black India ink; metastases were counted in dissected lung lobes under a stereoscopic microscope.

Molecular analysis of rat neu *gene presence and expression*

For genomic DNA extraction, 0.5×10^6 cells were pelleted, resuspended in 0.2 ml of extraction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.45% Tween 20, 0.45% NP40, and 0.12 mg/ml proteinase K) and incubated at 56°C for 90 min. Proteinase was inactivated by treatment at 95°C for 30 min. PCR was performed on 1 μl of DNA in a final volume of 50 μl. Primers to amplify vector-MMTV sequences were as described above in "Transgenic mice." Primers to amplify rat *neu* sequence were: 5′-AGGGCAACTTGGAGCTTACCTACG-3′ and 5′-GGGTTCTGCCTGGGGTGGGA-3′; these primers amplify a 234 bp product from the rat *neu* transgene, whereas they do not amplify endogenous murine *neu* sequence.

Northern blot analysis

RNA was extracted with RNAzol™ B isolation solvent (Tel-Test, Inc., Friendswood, TX) following the supplier's instructions. RNA (20 μg/sample) was electrophoresed in a 1% agarose-formaldehyde gel, transferred to a nitrocellulose filter (Schleicher and Schuell, Keene, NH) and immobilized by UV-crosslinking. Hybridization was carried out using a [^{32}P]dCTP (Amersham) probe, obtained by BamHI digestion of *neu* cDNA corresponding to 2248 bp of 3′ end excised from pRNeucDNA-H (kindly provided by Dr. P. Vezzoni), using a random-primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). After stripping, the membrane was hybridized with a control [^{32}P]dCTP β-actin probe (Oncogene Research Products).

Immunoprecipitation and Western blot analysis

Cells were trypsinized, washed twice with cold PBS and solubilized for 45 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, and 0.5% Triton X-100) containing protease plus phosphatase inhibitors, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM sodium orthovanadate (Na_3VO_4). Cell lysates were cleared by centrifugation at 4°C for 10 min at 10,000 g. Protein concentration was determined by the BCA protein assay (Pierce Biochemical Co., St. Louis, MO). Cell lysates (1.5 mg protein/sample) were immunoprecipitated after preclearing for 30 min with GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden) by incubation on a rocker with 3 μg/ml of monoclonal antibody 7.16.4 (Oncogene Research Products) or with mouse myeloma NSO-conditioned culture medium as negative controls for 3 hr at 4°C. Sepharose was added (20 μl) and after 3 hr incubation, immune complexes were washed three times with lysis buffer, eluted and denaturated by heating for 5 min at 95°C in reducing Laemmli sample buffer and resolved in a 7.5% polyacrylamide gel. Separated proteins were electrophoretically transferred to nitrocellulose membrane (Hybond C, Amersham, Little Chalfont, UK) and incubated at room temperature for 1 hr with anti-phosphotyrosine monoclonal antibody 4G10 (1.5 μg/ml; Upstate Biotechnology, Inc., Lake Placid, NY) and rabbit polyclonal anti-p185^{neu} serum C-18 (2 μg/ml; Santa Cruz Biotechnology, Inc.) followed by incubation with anti-mouse Ig and/or anti-rabbit Ig horseradish peroxidase-linked whole antibodies (1:10,000)

(Amersham) and visualized using the ECL detection system (Amersham) according to the supplier's instructions.

Analysis of in vitro cell growth and proliferation

Anchorage-independent growth in agar was determined by suspending 10^4–2×10^5 cells in DMEM + 20% FBS containing 0.33% agar; cells suspensions were then layered on a 5 ml base of 0.5% agar in 60 mm Petri dishes. Colony growth was monitored twice weekly and determined by counting at low magnification 14 days after seeding. In some experiments, cells were seeded in the presence of 1 μg/ml of anti-p185^{neu} monoclonal antibody 7.16.4 or of an isotype-matched antibody of unrelated specificity. For anchorage-dependent clonogenicity, 200–6,400 cells were seeded in 60 mm tissue culture Petri dishes in DMEM + 20% FBS. After 14 days, colonies were fixed in methanol, stained with Giemsa and counted with an inverted microscope at low magnification. Growth on plastic was studied by seeding 5×10^5 viable cells in 25 cm^2 flasks. Growth curves were obtained by direct count of cells harvested with trypsin-EDTA for 5 days after seeding. To determine saturation cell density, cells were grown to confluency and medium was renewed every 1–2 days thereafter; microscopic and visual inspection of cultures was carried out daily to exclude cell losses due to detachment from substrate. Cell yield was repeatedly evaluated over successive time points.

RESULTS

Expression of p185^{neu} in spontaneous mammary carcinomas of neu transgenic mice

Cytofluorometric analysis of 53 independent mammary carcinomas from 19 individual FVB-NeuN transgenic mice revealed consistently high levels of p185^{neu} expression on the tumor cell membrane (Fig. 1*a*). A narrow distribution of p185^{neu} expression was observed within each tumor (Fig. 1*b*), and no bimodal peaks suggestive of p185^{neu} loss variants were resolved. Immunohistochemical analysis of tumor specimens with an anti-p185^{neu} antibody confirmed the high level expression of the transgene in the mammary carcinomas (Fig. 2*b*).

In vitro selection of p185^{neu}-negative cells

A cell line, designated N202.1, was derived from one mammary carcinoma. Cytofluorometric analysis indicated high level expression of p185^{neu} by these cells but considerable heterogeneity of p185^{neu} expression in a series of clones randomly derived from N202.1, i.e., clone N202.1E expressed no detectable surface p185^{neu}, and two other clones (N202.1B and N202.1C) showed low expression (Fig. 3, left). All clones, regardless of p185^{neu} expression, shared the selective loss of H-2D^q expression (data not shown) previously found in mammary carcinomas of N202 origin (Lollini *et al.*, 1998), thus confirming a common neoplastic origin.

To determine whether the decline of p185^{neu} expression in these clones was due to the cloning procedure, we analyzed a series of long-term cultures established from independent transgenic mammary carcinomas (Fig. 3, right). One, designated TT3, had no detectable p185^{neu} expression similar to clone N202.1E, while a second, TT5, resembled clones N202.1B and N202.1C in the low level of neu expression. PCR analysis of genomic DNA with primers specific for rat *neu* and for sequences present in the vector originally used to generate the transgenic mice indicated the presence of the transgene in all clones, independent of cell surface p185^{neu} expression levels (Fig. 4*a*). *neu* mRNA was not detected in p185^{neu}-negative cells (N202.1E and TT3) by Northern blot analysis (Fig. 4*b*), suggesting control of expression at the transcriptional level. Western blot analysis of immunocomplexes from N202.1E and TT3 lysates revealed no p185^{neu} oncoprotein expression (Fig. 4*c*). p185^{neu} protein and its tyrosine phosphorylation were detected in N202.1A cells and, to a lesser extent, in N202.1B but not in N202.1E or TT3 cells (Fig. 4*d*).

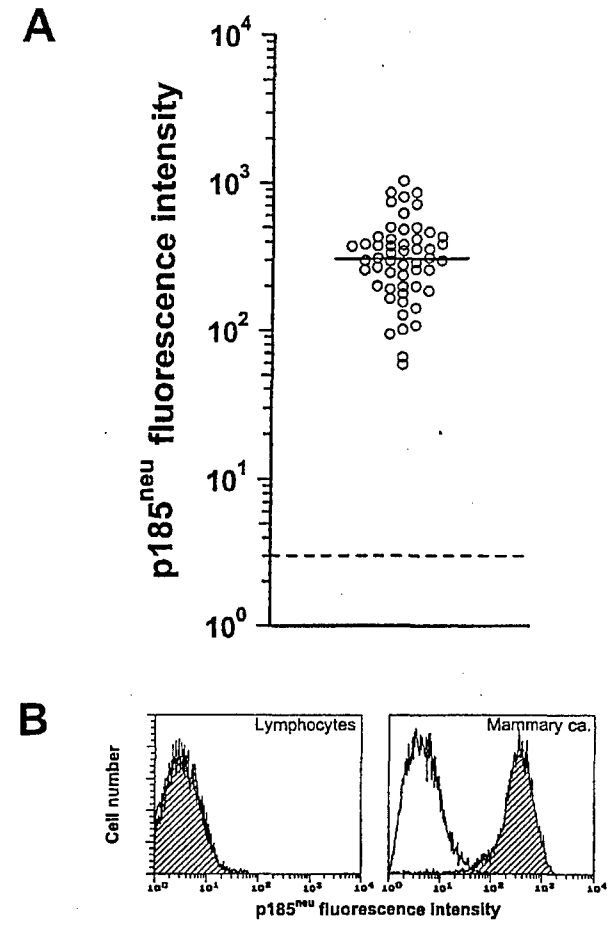

FIGURE 1 – Cytofluorometric analysis of p185^{neu} expression in enzymatically dissociated cells from mammary carcinomas of FVB-NeuN transgenic mice. *a:* Fluorescence intensity of p185^{neu} expression in 53 consecutive tumors. Continuous line represents the mean value of p185^{neu} fluorescence intensities. Dashed line represents the mean value of controls treated with secondary antibody alone. *b:* p185^{neu} expression in a representative mammary tumor (right) as compared with expression levels in normal T lymphocytes (left). Open curves: cells stained with secondary antibody alone; shaded curves: cells stained with anti-p185^{neu} antibody.

p185^{neu}-Negative cells give rise to dormant tumors

Comparison of the ability of p185^{neu}-positive and p185^{neu}-negative cells to grow as tumors in syngeneic transgenic mice indicated a very delayed onset of tumors generated from p185^{neu}-negative N202.1E and TT3 cells as compared with the short latency characteristic of p185^{neu}-positive N202.1A and N202.1B tumors (Fig. 5). Nevertheless, progressive tumors eventually appeared at the site of cell injection in almost all mice treated with N202.1E or TT3 cells, and the growth rate of established tumor masses was similar to that of N202.1A and N202.1B tumors (Fig. 5). Latency times showed by tumors induced by N202.1E or TT3 were almost always shorter than those observed for spontaneous tumors.

Similar analyses of tumorigenicity in athymic nude mice to exclude the interference of immune-mediated phenomena again revealed the pronounced delay in tumor appearance after p185^{neu}-negative cell injection as compared with p185^{neu}-positive cells: N202.1E-induced tumors appeared in 83% of nude mice with a latency time of 70–170 days, whereas all the mice receiving N202.1A cell injection became tumor-positive in 20–30 days.

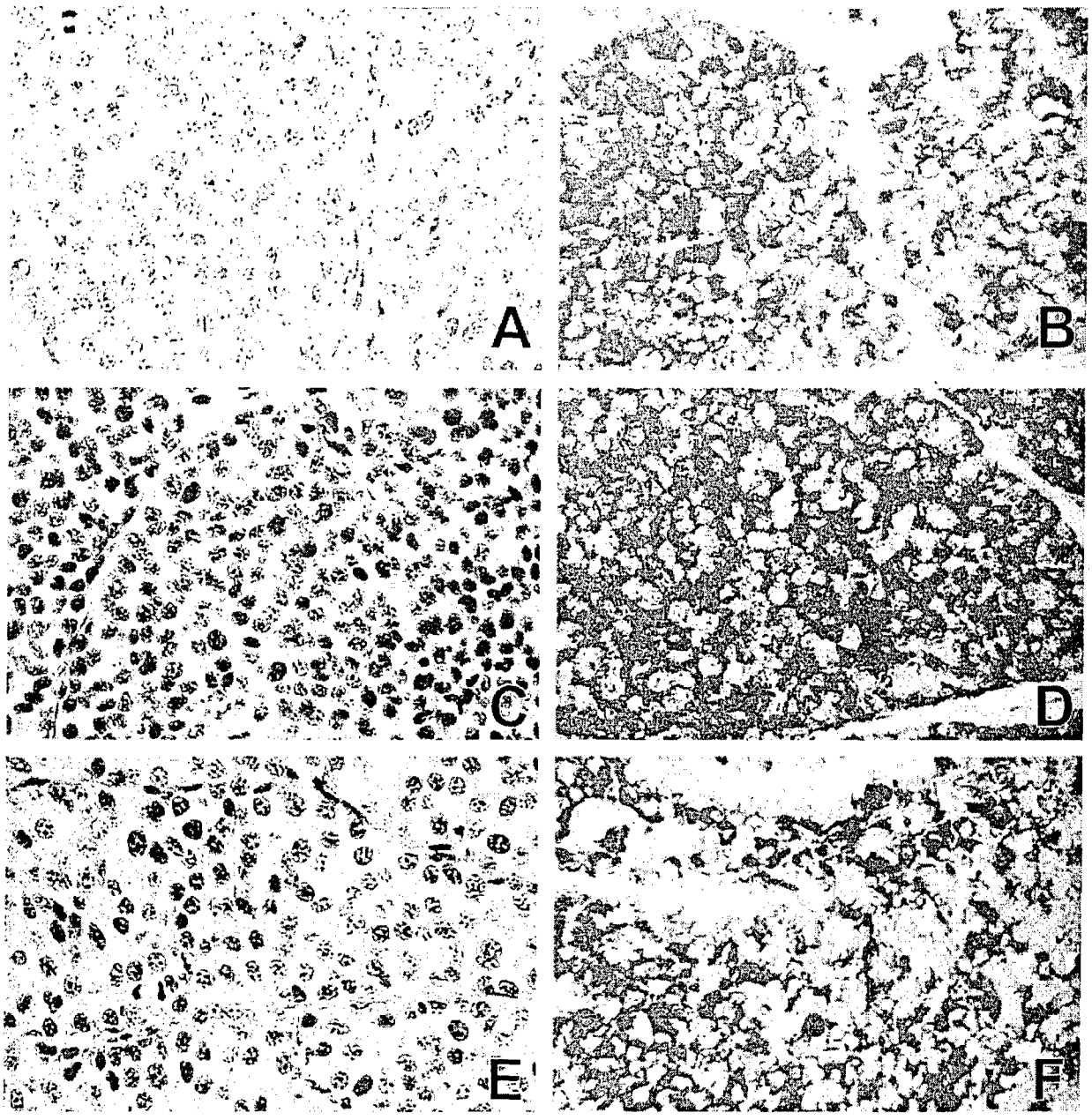

FIGURE 2 – Histological features (*a, c, e*) and immunohistochemical analysis for p185^{neu} expression (*b, d, f*) of mammary tumors grown in FVB-NeuN mice. *a,b:* spontaneous tumor; *c,d:* tumor induced by s.c. injection of N202.1A cells; *e,f:* long-latency tumor induced by s.c. injection of N202.1E cells. N202.1A and N202.1E cells both gave rise to a tumor morphologically similar to the spontaneous tumor. Anti-p185^{neu} immunostaining revealed an intense pattern of distribution of p185^{neu} expression in all tumors. *a,c,e*: hematoxylin-eosin, magnification: ×630; *b,d,f*: immunohistochemistry, magnification: ×630.

Experimental lung metastases were detected in all transgenic mice injected i.v. with N202.1A (median number of lung nodules = 76, range 60–111), whereas N202.1E cell-injected mice never developed metastases.

p185^{neu}-Negative cells induce p185^{neu}-positive tumors

The morphological features of N202.1E tumors were highly reminiscent of those found in primary tumors or in fast-growing tumors induced by p185^{neu}-positive N202.1A cells (cf. Fig. 2*e* with *a* and *c*). Immunohistochemical analysis of long-latency tumors arising after injection of p185^{neu}-negative N202.1E cells revealed a uniformly high expression of p185^{neu} (Fig. 2*f*). The intensity and cellular distribution of p185^{neu} staining were similar to those found in spontaneous primary tumors (cf. Fig. 2*f* with 2*b*) and slightly less homogeneous than that obtained with fast-growing p185^{neu}-positive N202.1A clone (cf. Fig. 2*f* with 2*d*).

Cytofluorometric analyses of tumor cells freshly dissociated from three mammary carcinomas produced by s.c. injection of clone N202.1E revealed in each case cells expressing high levels of p185^{neu} (Fig. 6). Long-term cell cultures that maintained high p185^{neu} expression over several *in vitro* passages were derived

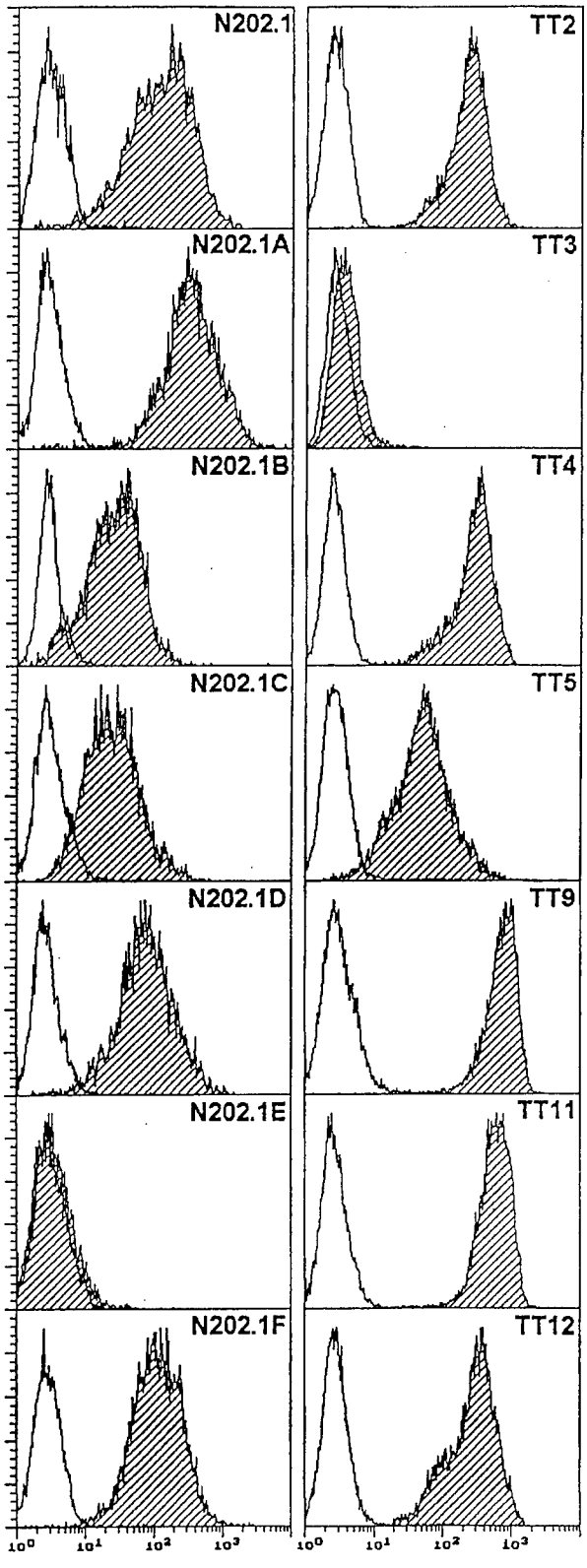

FIGURE 3 – Heterogeneous p185^{neu} expression in vitro among different established cell lines and clones derived from mammary tumors of FVB-NeuN mice. *Left:* N202.1 cell line and its clonal derivatives. *Right:* Cell cultures from independent mammary carcinomas. Open curves: cells stained with secondary antibody alone; shaded curves: cells stained with anti-p185^{neu} antibody.

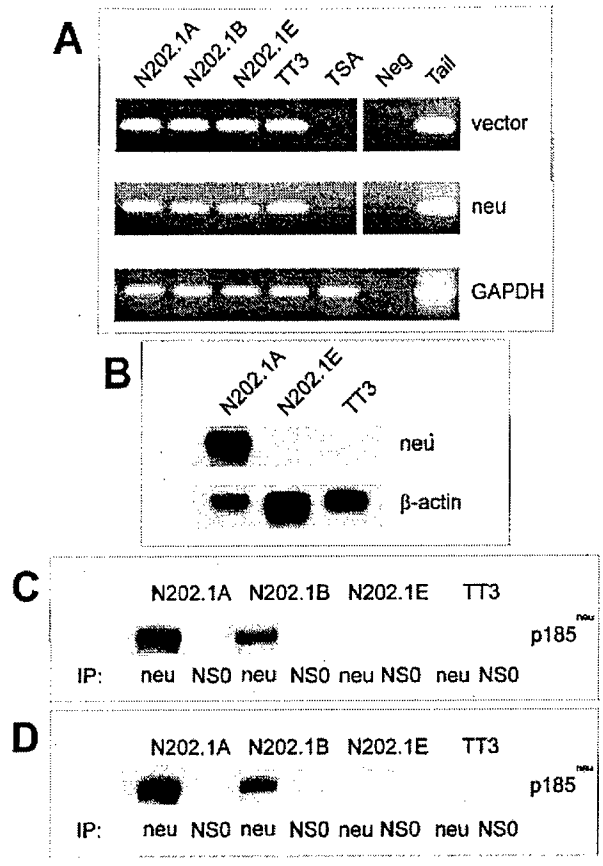

FIGURE 4 – Molecular analysis of *neu* transgene presence and expression in mammary carcinoma cell lines derived from FVB-NeuN mice. *a:* PCR analysis of genomic DNA from representative clones with high (N202.1A), low (N202.1B) or undetectable (N202.1E and TT3) cell surface p185^{neu} expression. Mammary carcinoma cell line TSA of BALB/c origin was included to show the species-specificity of rat *neu* primers, that do not amplify endogenous murine *neu* sequences. Controls: neg, no template; tail, positive control DNA extracted from tail of transgenic FVB-NeuN mice. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Amplified products: vector = 219 bp; neu = 234 bp; GAPDH = 452 bp. *b:* Northern blot analysis of *neu* expression. *c:* Western blot analysis of cell extracts immunoprecipitated with anti-rat p185^{neu} monoclonal antibody (neu) or with mouse myeloma NSO-conditioned culture medium as negative control. *d:* Immunoblot analysis of p185^{neu} phosphorylation in cell extracts immunoprecipitated with anti-rat p185^{neu} monoclonal antibody (neu) or with mouse myeloma NSO-conditioned culture medium as negative control.

from two such tumors. One of the cultures, designated 1E-neu+, was used for s.c. injection in syngeneic transgenic mice; tumors arose with a short latency time, unlike those observed after N202.1E injection (Fig. 5).

p185^{neu} Expression favors anchorage-independent growth but hampers anchorage-dependent growth

Current tumorigenicity data suggest that p185^{neu} expression confers a growth advantage to mammary carcinoma cells that could be mediated either by host-dependent interactions (*e.g.*, neoangiogenesis) or by intrinsic growth properties of tumor cells. To investigate this issue, we compared the *in vitro* growth of p185^{neu}-positive and p185^{neu}-negative mammary carcinoma cells.

Anchorage-independent clonogenicity was first analysed. N202.1A and 1E-neu+ cells were able to form large colonies in soft agar, while N202.1E produced no colonies at any cell concentrations tested, and TT3 cells formed only rare small clusters

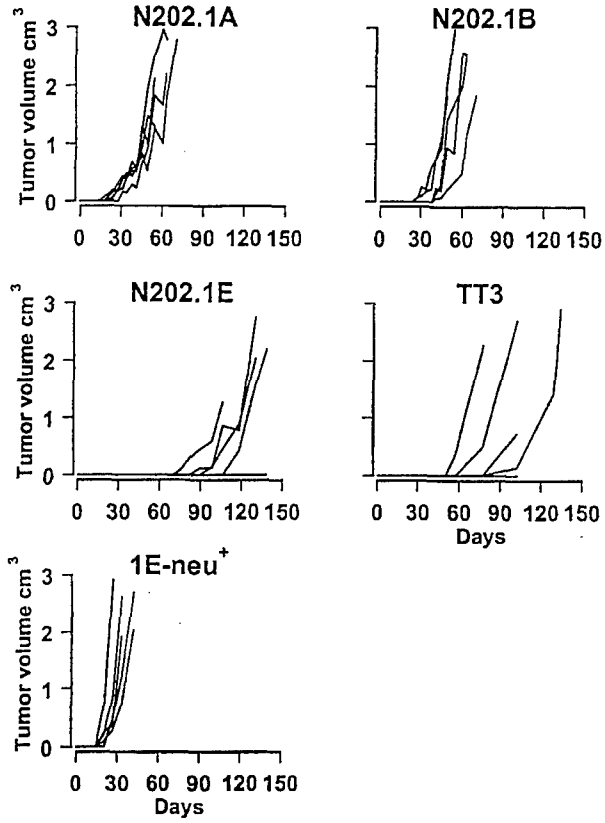

FIGURE 5 – Tumor growth of different transgenic mammary carcinoma cell lines injected s.c. into syngeneic FVB-NeuN mice. Tumor volumes are shown for each of 5 mice per group. Data are representative of at least 2 independent experiments.

(Figs. 7*a*, 8). Moreover N202.1A growth in agar was significantly inhibited in the presence of an anti-p185^{neu} monoclonal antibody but not of an irrelevant antibody (Fig. 7*b*).

On the contrary, anchorage-dependent growth of p185^{neu}-negative N202.1E and TT3 cells was significantly more robust than that of p185^{neu}-positive N202.1A and 1E-neu+ (Fig. 7*a*), explaining the relative ease with which p185^{neu} loss variants emerged during *in vitro* culture. The better adherent growth of p185^{neu}-negative cells was not determined by a different adhesion ability, as evaluated 24 hr after cell seeding (data not shown) but resulted from a higher clonogenic growth on plastic surfaces (Fig. 7*a*) and from higher proliferation rates, as also revealed by bromodeoxyuridine labeling (data not shown). However, p185^{neu}-positive cells continued their growth after confluency and reached saturation densities higher than those of p185^{neu}-negative cells (Fig. 7*a*). This fact could be related to a more differentiated pattern of growth with dome formation observed in adherent cultures of p185^{neu}-negative N202.1E and TT3 cells.

DISCUSSION

In the present study, we show that the oncogenic activity of p185^{neu} contributes to the transformed and tumorigenic phenotype, not to cell proliferation of transgenic mammary carcinomas. In fact, overexpression of p185^{neu} in our murine system appeared to increase anchorage-independent growth but negatively affect anchorage-dependent cell proliferation and clonogenicity. The better adherent growth of p185^{neu}-negative cells could be related to molecules involved in adhesion phenomena and down-regulated in p185^{neu}-positive cells such as galectin-3 (data not shown).

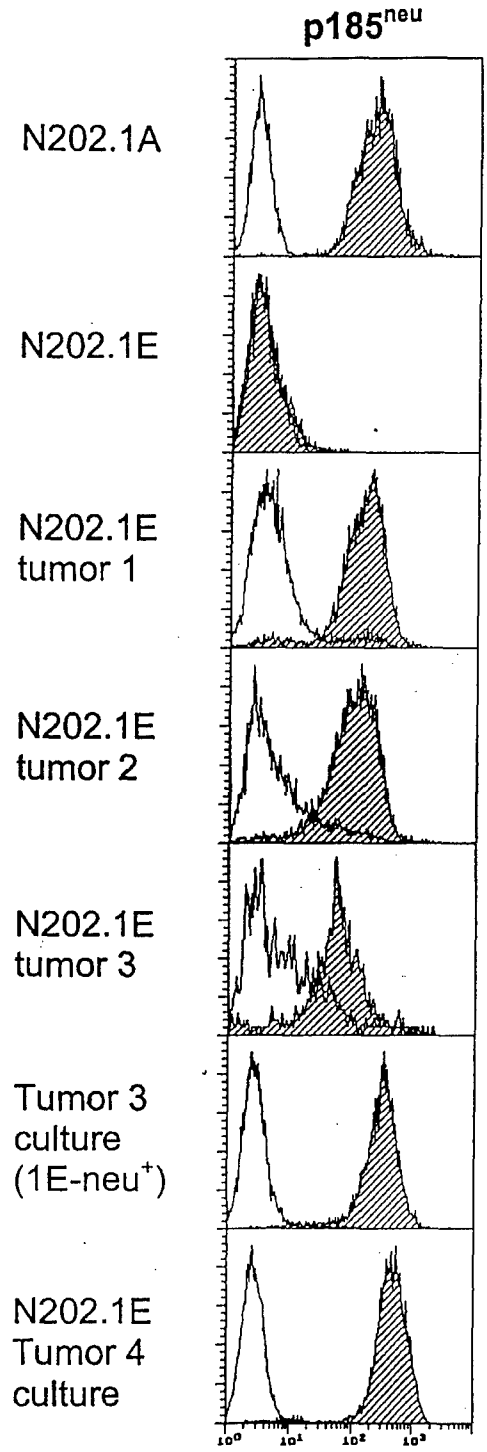

FIGURE 6 – Cytofluorometric analysis of p185^{neu} expression in long-latency tumors induced by s.c. injection of p185^{neu}-negative N202.1E cells and derived cultures. Open curves: cells stained with secondary antibody alone; shaded curves: cells stained with anti-p185^{neu} antibody.

Expression of p185^{neu} was apparently required for tumorigenicity and metastatic spread, since rapid tumor formation was observed only with p185^{neu}-positive cells, and all tumors that eventually arose from p185^{neu}-negative cells were invariably p185^{neu}-

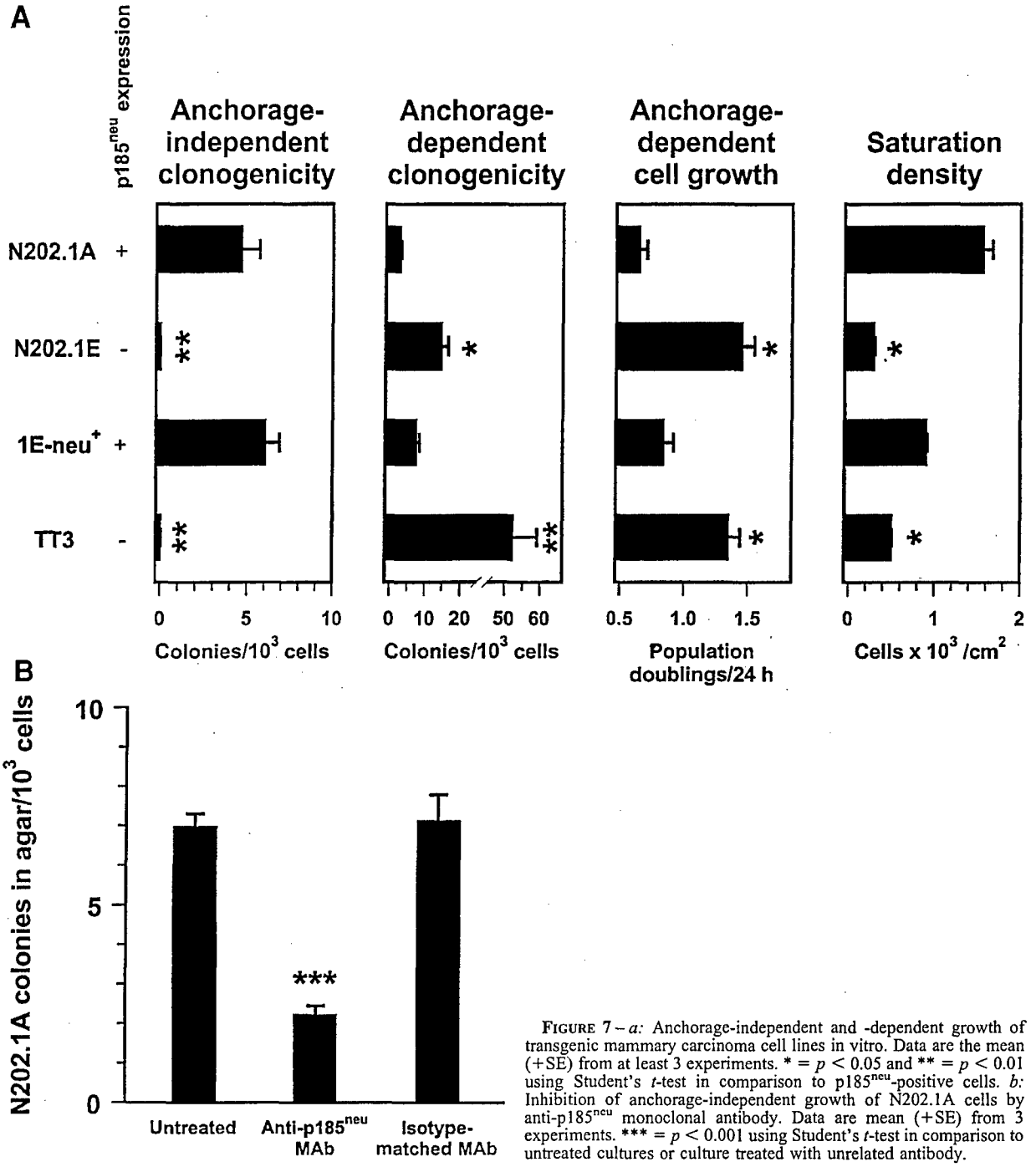

FIGURE 7 – *a:* Anchorage-independent and -dependent growth of transgenic mammary carcinoma cell lines in vitro. Data are the mean (+SE) from at least 3 experiments. * = $p < 0.05$ and ** = $p < 0.01$ using Student's *t*-test in comparison to $p185^{neu}$-positive cells. *b:* Inhibition of anchorage-independent growth of N202.1A cells by anti-$p185^{neu}$ monoclonal antibody. Data are mean (+SE) from 3 experiments. *** = $p < 0.001$ using Student's *t*-test in comparison to untreated cultures or culture treated with unrelated antibody.

positive. *In vitro* studies revealed that $p185^{neu}$-dependent tumorigenicity depended on the ability to grow anchorage-independently and to reach high saturation densities, rather than on superior cell proliferation. It is well known that anchorage independence and growth to high densities are related to tumor formation by transformed cells, and our results are in good agreement with earlier findings obtained by gene transfection of *neu* into NIH3T3 fibroblasts (Baasner *et al.*, 1996; Di Fiore *et al.*, 1987), which acquire tumor-forming ability in parallel with *in vitro* growth properties similar to those controlled by $p185^{neu}$ in our transgenic mammary carcinoma system.

In the context of normal mammary tissue, the growth autonomy conferred by $p185^{neu}$ expression might be related to discrete developmental stages in which anchorage-independence and growth at high cell density are required. In pregnant rats, an increase in $p185^{neu}$ expression was observed during the last steps of functional development of the normal mammary gland (Kokai *et al.*, 1987). In human prepartum and lactating mammary specimens, $p185^{HER-2}$ expression was detected at an intensity comparable to that of breast carcinomas (Kacinski *et al.*, 1995). In another study, transfection of *HER-2* gene into MCF-7 breast carcinoma cells appeared to increase differentiation of these cells (Giani *et al.*, 1998).

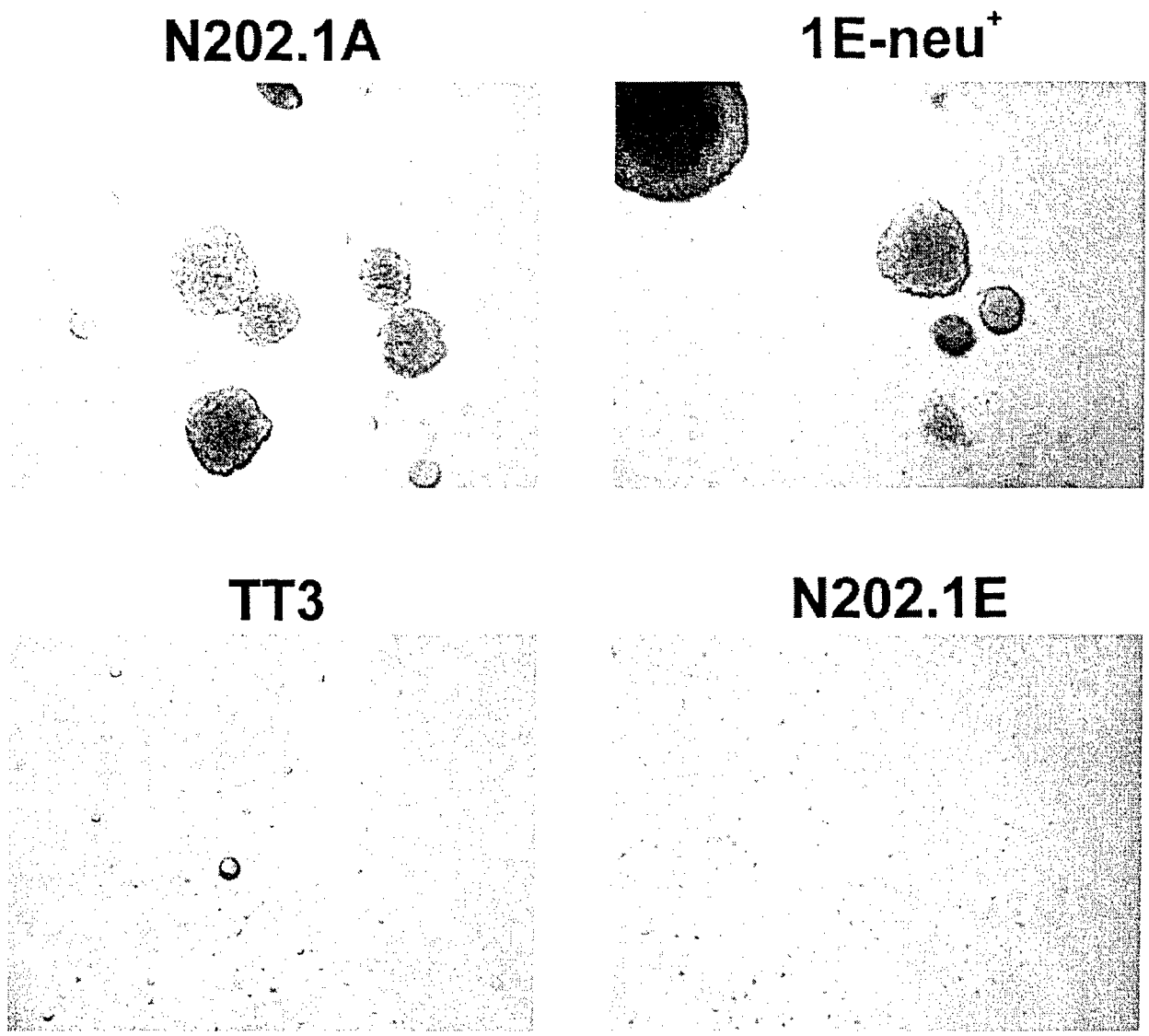

FIGURE 8 – Soft agar cultures of transgenic mammary carcinoma cell lines positive or negative for p185^{neu} expression. Magnification: ×25.

The pattern of tumor growth of p185^{neu}-negative cells, observed in our study, *i.e.*, a long latency period followed by the emergence of a fast-growing variant, is reminiscent of the behavior of dormant tumors (Demicheli *et al.*, 1994). Colony formation in soft agar was completely predictive of the ability to form rapidly growing tumors. The molecular events that determine dormancy and the restart of tumor growth in human breast carcinoma are not known but current hypotheses include mechanisms based on angiogenesis and interaction with the immune system (Uhr *et al.*, 1997). We suggest that changes in the level of p185$^{HER\text{-}2/neu}$ expression might be causally related to variations in the dormant status of breast carcinoma.

In conclusion, our results indicate that p185$^{HER\text{-}2/neu}$ expression is not required for the unrestricted proliferation of mammary carcinoma cells but is indispensable for specific steps of progression involving anchorage-independent growth of tumor cells, which determine the ability to form aggressive and metastatic tumors.

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The prospects for cancer gene therapy

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Abstract

Gene therapy is a flexible technology with which to look for new ways of inhibiting cancer. However, the marginal success achieved has made it clear that direct engineering of cancer cells is more complex than had been supposed. The main barriers are raised by the difficulty of securing gene delivery into cancer cells in vivo and the selective advantages of those against which it is ineffective. These drawbacks do not arise when an immunological approach is adopted. Genes coding for tumor-associated peptides are used to engineer professional antigen presenting cells (APC). Alternatively APC pulsed with tumor antigens are engineered to overexpress costimulatory molecules or release cytokines. A more conservative approach is to engineer whole tumor cells with costimulatory and MHC molecules. Tumor cells can also be engineered to secrete cytokines and chemokines. The sustained presence of these factors in the tumor microenvironment recruits and activates distinct repertoires of APC and skews the antitumor response towards Th1 or Th2 reactivity. Engineered tumor cells are quickly rejected while mice acquire an immune memory against subsequent challenges, even when the tumor involved is poorly immunogenic. They also cure mice bearing incipient tumors and small metastases. This efficacy, however, vanishes as the tumor progresses. Even the best-induced specific immunity, therefore, is of no avail against advanced tumors. By contrast, the experimental data endorse the rational expectation that cancer vaccines will soon be both an established treatment of minimal disease after conventional management and a way of securing preventive antitumor vaccination.

1. Introduction

Recombinant DNA technologies have unexpectedly extended the frontiers of biology well beyond those that once seemed so clearly defined. Transgenic foodstuffs, genetically modified plants and animals, and drugs created by genetic engineering are examples of the apparently harmless achievements through which biology is radically changing our everyday lives. Any ordinary laboratory, in fact, can now isolate genes, alter them in vitro and reinsert them in vivo, and build plants and animals with a modified or even a partly novel genetic complement.

Rational evaluation of these new and to some extent unforeseen opportunities is hampered by the uneasiness that both the products of recombinant technology and above all the possibilities that lie before it commonly arouse. One's mind

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Appendix 7

naturally turns to Dr Frankenstein and his monster [1]. Uncertainty and even fear inevitably accompany every attempt to predict the behavior and ecological consequences of the seemingly docile and very useful monsters now issuing from the womb of genetic engineering [2].

2. Gene therapy

The service currently rendered to medicine by genetic engineering can be briefly stated as the permanent endowment of a cell carrying a gene that is broken or inactive and hence the cause of a disease with the same gene in proper working order. This possibility is obviously of particular assistance in the treatment of genetic disorders, such as congenital immunodeficiencies. Much work has been done in this field. Lymphocytes with an altered gene harvested from a patient and cured in vitro by insertion of the corresponding normal gene should function properly when re-inoculated and have a selective advantage over their untreated fellows [3].

This paradigmatic approach, however, is fraught with difficulties. There are still very few diseases for which it is correct to speak of a cure based on gene therapy. In other cases, attempts are made to deliver the operating gene directly into the host organs and tissues expressing the broken gene [4].

3. Treatment strategies

A very different situation must be faced in oncology. Here it is no longer a question of adjusting cells struggling to survive and proliferate because they carry an altered gene. On the contrary, their genetic alterations are the very reason why neoplastic cells can proliferate ad lib regardless of the control mechanisms that keep normal cells within bounds. This, indeed, is why tumors form and may go on to colonising other organs. Blocking the unbridled proliferation and dissemination of neoplastic cells is no easy task, even with the aid of genetic engineering.

The matter is further complicated by the fact that acquisition of a malignant invasive and metastatic phenotype takes place in several stages through the accumulation of indepedent gene alterations. For some types of tumors, specific molecular progression models embracing alterations more frequently associated with the initial stages (such as the transition between preneoplastic forms and benign tumors) and others often associated with such acquisition have been proposed. The tumor cell to be treated is often a 'multiple genetic syndrome' and as such requires an approach employing several strategies, including the combination of conventional management and gene therapy.

For this reason, strategic approaches widely differing in both conceptual and technological terms are being currently considered within gene therapy itself. One cannot tell whether a single strategy will prevail and be normally used, or whether different approaches will be applied to different clinical situations. Five of the main approaches are described below.

3.1. The missionary approach

This term refers to the insertion in neoplastic cells of a gene that inhibits their malignant phenotype and 'converts' them to normal ways of behaving. The basic premise for the application of this approach is identification of the gene alterations that play key roles in specific types of tumor.

The alterations that lead to the malignant phenotype fall into two conceptual categories: alterations that result in a loss of function (oncosuppressor genes) and those that result in a gain of function (oncogenes). Gene therapy must obviously restore the function lost through inactivation of an oncosuppressor gene in the first case and suppress the function newly acquired through activation of an oncogene in the second.

Preclinical animal studies of the reconstitution of oncosuppressor genes have provided encouraging results. Gene therapy targeted to p53, for example, has already formed the subject of completed clinical trials. In vitro studies, in fact, had shown that transduction of a wild-type p53 gene

blocked the proliferation of nearly all tumor cells with p53 mutation and often induced their apoptosis. Since malignant cells are the product of multiple hits, the importance of this result lies in the demonstration that their uncontrolled proliferation can be halted by normalising just one of the genes involved in tumor progression. p53 controls the expression of numerous growth factors and cytokines that autocrinally and paracrinally stimulate tumor growth and angiogenesis. Transduction of wild-type p53 is thus followed by bystander effects through its diminution of the supply of factors that promote the growth of the non-transduced cells. This suggests that 100% transduction efficiency in vivo is not an essential requirement [5].

The retroviral transduction vectors used in the initial clinical studies have since been replaced by adenoviral vectors. The tumors treated have usually been lung and head and neck carcinomas. The vectors are introduced via a bronchoscope or directly injected into the tumor mass. Complete and partial regressions have been reported [6,7]. A systemic effect, however, has always failed to materialise.

Ablative gene therapy to neutralise activated oncogenes relies on the introduction of antisense sequences to neutralise the altered gene or its RNA [8]. Oncogene products can also be blocked by insertion of genes that code for the antigen binding regions derived from intracellular monoclonal antibodies (known as intrabodies) specific for the target protein that then directly inhibit its function and in effect act as antisense proteins [9].

Many new non-genic forms of treatment are now competing with these approaches. Inhibitors designed to block oncogenes with tyrosine kinase activity and some inhibitors of farnesylation of the RAS oncogene are particularly promising [10]. Their advent may mean that gene therapy will not prove to be the most effective way of inhibiting many oncogenes.

3.2. Mega-lysis

Some viruses known as oncolytic viruses only replicate, whether spontaneously or as the result of genetic modifications, in neoplastic cells and are being used for their selective destruction, since the surrounding normal cells are spared. An adenovirus modified in the protein E1B so as to be only able to replicate in cells with p53 alterations is in the most advanced stage of development. During its replication, p53 interprets its DNA as a damage signal and blocks both cellular and viral replication. The virus can only replicate if E1B blocks p53. E1B gene mutations or losses corresponding to the p53 interaction region thus produce an adenovirus that can only replicate in cells devoid of a functional p53, as are many tumor cells [11].

3.3. Metabolic suicide

This approach requires the delivery to a neoplastic cell of a gene that will make it selectively susceptible to drugs that have no effect on non-engineered cells. The genes concerned code for many enzymes that belong to the microbial world and convert certain nontoxic prodrugs into toxic metabolites. The two enzymes most commonly employed in tumor management are cytosine deaminase, which metabolises 5-fluorocytosine, and thymidine kinase, which metabolises Ganciclovir [12,13]. Cells that acquire these genes grow and behave in the same way as normal cells. The difference between them, however, lies in the fact that normal cells cannot metabolise the prodrug and continue to live undisturbed, whereas the engineered cells do metabolise it because of their acquired gene and are selectively killed by its toxic metabolites in a form of metabolic suicide.

This approach is especially attractive on account of its bystander effect, whereby the suicide of many engineered cells induces the death of an often substantial number of non-engineered cells. As in the case of the missionary approach, therefore, transduction of the metabolic suicide gene into every neoplastic cell may not be an essential prerequisite for tumor eradication.

It should also be noted that the enzymes employed are not present in mammals. By acting as neoantigens, therefore, they might stimulate an immune response against non-engineered cells as in the immunological approach described in Section 3.6 below. Participation of such a response in

the bystander effect has, in fact, been observed in some experimental systems [13,14]. At present, however, it is not clear how far this effect is generalisable, nor whether the metabolic suicide approach can be worked up to the point of becoming a new and effective form of treatment.

3.4. The antiangiogenic or Dracula approach

A solid tumor needs new vessels to be able to grow and their number is often correlated with its invasive and metastatic capabilities. It activates angiogenesis by locally upsetting the balance between numerous angiogenic and antiangiogenic factors. For this purpose, it often produces angiogenic factors itself or induces their production on the part of fibroblasts and endothelial cells, as well as those of the local inflammatory infiltrate. The aim of antiangiogenic management is to reduce the growth and metastasisation of a tumor by diminishing its vascular network [15]. Its targets are the tumor cells, the endothelial cells, the extracellular matrix or the proteolytic enzymes. Preclinical studies using mouse tumors or human tumors grown in immunodepressed mice have shown that their angiogenesis and growth and the metastasisation of their cells can often be reduced by the repeated inoculation of antiangiogenic proteins. Frequent repetition of high doses is necessary, however, and the factors employed induce numerous side effects. Suspension, too, is followed by the resumption of tumor growth [16].

An effective antiangiogenic treatment must obviously be able to oppose the proangiogenic phenotype of the tumor. At the same time, however, it must not interfere with physiological angiogenesis. Gene therapy has been investigated as it seems best able to meet these two main requirements. The aim is to secure lasting expression of high levels of antiangiogenic molecules in the tumor area without resorting to high systemic doses. The risk of side-effects is reduced because selective gene expression in the target area can be achieved by choosing appropriate viral vectors (retroviruses, for example, which preferably integrate in proliferating cells and hence those of the tumor, and in those of its endothelium, which proliferate 50 times more than normal endothelium cells), and the use of organ- or tissue-specific promoters.

By comparison with other gene therapy prospects, the antiangiogenic approach has the advantage of also inducing a marked effect on non-engineered cells. Irrespective of the cells that produce them, angiogenesis regulators merely need to be present in the zone where they have to act and transduction of a particular type of cell or all the target cells is not required. Following transduction of the gene of an antiangiogenic factor into mouse mammary tumors, for example, less than 5% of cells were transduced, whereas reduction of the blood vessels exceeded 60%. Resistance and clonal selection do not seem to arise, since the endothelial target cells are normal and should not display the genetic instability characteristic of tumor cells.

3.5. The protective approach

This envisages the transfer to normal bone marrow stem cells of genes that will make them resistant or less sensitive to the antiblastic and cytotoxic drugs regularly used in tumor therapy. Marrow cytotoxicity, in fact, limits the doses that can be employed. Greater blast resistance induced by genetic should thus overcome this barrier and allow significant dose escalation. Experiments conducted with rats, dogs, sheep and monkeys have shown that bone marrow and peripheral stem cells can be transduced with such genes and reinfused to colonise the marrow. Preliminary data suggest that this approach is feasible in humans. What remains to be determined, however, is whether the employment of significantly higher doses will be limited by the onset of other types of toxicity in non-hematopoietic organs. A close study must also be made of the long-term consequences particularly the frequency of the neoplastic transformation of transduced stem cells [17].

3.6. The immunological approach

This approach obviously sets out from very different theoretical premisses. Its aim is to activate an immune response powerful enough to interfere with a tumor's growth. Genetic engineering, therefore, is simply a way of trying to achieve

the success that has eluded other methods over the course of many years. Its armamentarium is composed of two new weapons: super killer cells and antitumor vaccines.

3.6.1. Super killer cells

Extremely efficacious and selective killer cells can hopefully be constructed for use against frank and massive tumors. Numerous experimental and clinical data have shown that natural killer cells activated in the presence of IL-2 (LAK cells) sometimes induce dramatic and persistent regression of such tumors [18]. This technique, however, has been gradually abandoned. Its implementation, in fact, is complicated and unwieldy, while its results are both sporadic and unpredictable. An attractive and logical alternative, namely the engineering of super killer cells that are not aspecifically activated by IL-2, but genetically constructed for the specific and highly efficient slaughter of tumor cells, is being investigated by some laboratories [19].

3.6.2. Antitumor vaccines

Here the objective of genetic engineering is to produce intelligent vaccines endowed with the requisites that basic research has progressively shown to be necessary for the mounting of an effective immune response, and thus do away with the need for its indirect induction on the part of antigen presenting cells (APC). Dozens of rodent and human tumor cell clones have now been engineered to express a wide range of proteins that influence the immune response on their membranes, namely costimulation, self-histocompatibility and allogenic histocompatibility molecules, and the products of metabolic suicide genes [13].

It has also been shown that tumor cells engineered to secrete cytokines become much more immunogenic. Their proliferation, in fact, is accompanied by the pumping out of increasing amounts of cytokines that often activate a primarily inflammatory response and thus lead to rejection of the engineered tumor. Every cytokine, however, activates a different and usually very complex reaction governed by the interaction between specific and nonspecific immunity cells [20].

Rejection of cells engineered to release cytokines often establishes a systemic immune memory with the result that a subsequent potentially lethal challenge with non-engineered parent cells is also rejected. The mechanisms underlying this memory vary from one cytokine to another. Some cytokines mainly induce Th1-type responses, others induce Th2-type responses [20]. There are also reports of cases in which the memory thus established is equally capable of slowing down, if not blocking, the growth of existing tumors [21]. Furthermore genetic diagnosis is now beginning to identify normal persons with an inherent risk of developing a cancer against which they could perhaps be effectively vaccinated [22].

An alternative strategy for the construction of antitumor vaccines is to engineer professional APC in such a way as to render their presentation of the tumor-associated antigens, identified and both molecularly and genetically defined in numerous human and animal tumors, particularly effective.

4. Possible and impossible immunological objectives

The experimental evidence set out above shows that genetic engineering can greatly increase the immunogenicity of tumor antigens. This increase, too, is fully in line with the theoretical premisses behind the immunological approach. Is it, however, sufficient to cure tumors by vaccination with engineered cells? The experimental data, indeed, seem to shy away from providing a satisfactory answer when they indicate that:

1. Engineered cells evoke an immune reaction that is often strong enough to result in their rejection. However, it is the engineered cells alone that both induce the rejection and are themselves rejected;
2. This rejection often establishes a strong immune memory that is specific for both the engineered cells and their unmodified parent cells. This is naturally important because it represents an efficient way of immunisation against tumors. But who is to be immunised? In the experimental scenario, the subject is a

fully normal and healthy mouse that is immunised and then very effectively rejects a subsequent tumor challenge.

We are thus able to mount a strong response giving protection against aggressive, poorly immunogenic tumors. The data, however, stop short of suggesting that tumors can be cured. It is not that the question of curing tumors has been ignored. Unfortunately the results of the experiments that set out to address it are far from satisfying. A good example is provided by direct investigations using TSA cells from an aggressive mouse mammary carcinoma that reproduces some aspects of human breast tumors. Immunisation with variously engineered TSA cells protected mice against subsequent potentially lethal challenges with the parent cells. It failed to cure those with a clinically detectable tumor, whereas many with minimal disease after tumor surgery or with early lung metastases were cured. All the data in the literature, indeed, show that vaccination is powerless against a clinically evident tumor. It loses its effectiveness as the tumor mass becomes larger and more organised [21,23].

Extrapolation of these findings to human tumors is hampered by the fact that the transplantable mouse tumors regularly employed grow very quickly to form extensive, incurable masses. Human tumors grow much more slowly. There may thus be an interval during which the immune reaction elicited by vaccination might be able to impede their growth.

It is clear from the experimental data, therefore, that genetic engineering provides vaccines inducing a specific immune response that is efficacious against minimal residual disease, but gradually loses its effectiveness for two reasons. In the first place, as indicated earlier, a tumor's proliferation kinetics can outpace the destructive capabilities of the immune system. In the second place, it forms an extracellular matrix that the specific immune attack cannot penetrate. Lymphocytes are held back by the barrier of the tumor's stroma and cannot even exit from its supply vessels. Even when they do get inside a tumor, they are paralysed by the immunosuppressive factors released by its cells [24]. It can thus be concluded that genetic engineering applied to antitumor vaccination has attractive prospects, provided an immune response is induced to prolong the disease-free interval of patients whose tumor mass has been greatly reduced by surgery, drugs or radiation.

Other applications are suggested in the light of what is now known about the dissemination and the even long residence of non-proliferating, dormant metastases and the elaboration of sensitive techniques for the identification of their bearers [25]. Chemotherapy is usually designed to attack actively proliferating cells and is presumably ineffective against these dormant metastases, whereas they could be an ideal target for the immune reaction. If employed in association with ad hoc conventional protocols, therefore, gene therapy could extend the treatment coverage to include cells not actively proliferating.

5. Prospects for the immune prevention of tumors

By comparison with conventional antitumor management, vaccination with engineered tumor cells is a soft, noninvasive treatment and free from particular inconveniences. If, as is probable, it proves effective in the management of minimal residual disease, it will result in radical changes in a patient's quality of life and life expectancy. To achieve this a series of obstacles must be overcome. In the first place, an assessment must be made of the real effectiveness of vaccination in minimal residual disease situations. Courage is required to set up randomised trials embracing large number of patients in these conditions. The scientific community is reluctant to embark upon such studies because they are complex, costly and very long. It would be important to manage to establish intermediate points indicating the progress of the clinical situation, since the desire to study the evolution of the disease in conditions relatively good for the patient means that one may have to wait several years for the appearance of any tumor recurrences.

The notion that one is affirming with regard to the efficacy of vaccination, in fact, is that the better the state of the vaccinated patient, the greater the probabilities that vaccination will

manage to control his residual disease. This concept, taken to extremes, leads to the conclusion, typical of preventive medicine, that vaccination is particularly effective when a person is not yet a patient, but a normal individual with only a greater than average risk of developing a particular neoplasia. Vaccination of persons at risk using antitumor vaccines prepared by genetic engineering is a new and attractive possibility. In numerous communities, women are encouraged to take tests for the early diagnosis of uterus and breast tumors. These screenings often reveal preneoplastic lesions indicating a high risk of developing a tumor. Other communities provide genetic testing to look for mutations that predispose to breast tumors. In this case, too, the bearer has a notably elevated risk of developing tumors that will present a characteristic antigen pattern that is, to a great extent, foreseeable. Another situation of risk in the absence of disease is that of patients cured for one tumor and likely to develop a second. These three situations give an idea of what could be the ideal subjects for preventive vaccination in the absence of disease [23].

Vaccines constructed by genetic engineering will have to present the antigens that will be expressed by tumors that have not yet appeared. This paradoxical situation, however, can be dealt with and solved on technological grounds, considering that one knows more and more what are the tumor antigens commonly expressed on the membranes of the neoplastic cells of that given type of tumor. In addition, the preparation used as a vaccine could contain more than one tumor antigen. Moreover, in various cases, mutations of the oncogenes that are involved in tumor onset may themselves code for proteins that can act as tumor antigens.

6. Conclusions

Completed clinical studies have shown that gene therapy is feasible and relatively free from toxicity. The results obtained so far, however, are mainly confined to localised tumors. No systems exist as yet for the systemic transduction of therapeutic genes, for example in cases of metastatic disease, where the immune approach is the best because it exploits the ability of the immune system to act at the systemic level. Here, however, the first clinical studies have shown a marginal and still not well defined therapeutic efficacy. The immunological conclusions, on the other hand, are different. In this case, genetic engineering, rather than opening new horizons, constitutes an extraordinarily flexible technique for reaching objectives pursued for more than 50 years. A rational definition of the objectives that can be reached with the different strategies will be able to ensure that in the near future immunotherapy becomes a new therapeutic modality. It is unlikely that the results will be revolutionary or lead to the curing of clinically frank tumors. More probably, genetic engineering applied to immunotherapy will be able to lead to interesting results by significantly lengthening the disease-free interval both in not-yet patients with a genetic risk of tumor and in patients at risk for tumor recurrences.

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The intriguing role of polymorphonuclear neutrophils in antitumor reactions

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Polymorphonuclear neutrophils (PMNs) are the most abundant circulating blood leukocytes. They provide the first-line defense against infection and are potent effectors of inflammation. In addition, their release of soluble chemotactic factors guides the recruitment of both nonspecific and specific immune effector cells.[1] Finally, since they both respond to and produce cytokines,[2,3] they also modulate the balance between humoral and cell-mediated immunity by contributing to the promotion of a T_H1 or T_H2 response.[4] In these ways, PMNs are engaged in a complex cross-talk with immune and endothelial cells that bridges innate and adaptive immunity.[5]

Even though many facets of their biology have been thoroughly investigated,[6] PMNs still have every reason to complain of the disdain with which they are regarded by oncologists and immunologists.[2] So widespread is T-cell chauvinism[7] that the antitumor potential of PMNs continues to receive little attention, and researchers have not yet fully considered the possibility of exploiting their functions as effective weapons against cancer.

Natural antitumor activity

The attempts of leukocytes to respond to cancer are suggested by their systemic, regional, and intratumoral activation.[8-10] Infiltration of tumors by leukocytes has been associated with a favorable prognosis in some studies in humans.[11-13] However, for individual patients, there is no predictable relationship between leukocyte composition and the prognosis of their disease.

PMNs are usually a scarce reactive component of both human and animal tumors. In animal models, their presence may sometimes be detrimental by favoring malignant growth and progression.[14] Nevertheless, recent studies have suggested that they are active in immunosurveillance against several tumors.[15-18] These intriguing outcomes are probably related to the result of the interplay between (1) the kind and amount of cytokines and chemotactic factors naturally released by tumor cells[19] and (2) the degree of recruitment and activation of the intermingled PMNs.

Over the last decade, cytokine gene transfer strategies in animal models have provided a tool with which to dramatically increase intratumoral cytokine availability, avoid the side effects of systemic administrations, and evaluate the antineoplastic potential of locally recruited PMNs.

Cytokines at the tumor site

The natural tumor-PMN balance can be markedly altered by engineering tumors to release interleukins[20] or chemokines[21] in their microenvironment. Although the amount released is usually small, it may be gigantic when compared with wild-type tumors and produce dramatic effects.

Almost all the cytokines sustainedly released by engineered tumor cells, namely interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-7, IL-10, IL-12, interferon-α (IFN-α), IFN-β, IFN-γ, granulocyte-colony stimulating factor (G-CSF), and tumor necrosis factor-α (TNF-α),[22,23] quickly recruit a massive local reaction that leads to the rejection of engineered tumor cells and the establishment of a significant immunity against the wild-type parental tumor. PMNs play a key role in all of these cytokine-induced tumor rejections, often in cooperation with $CD8^+$ T lymphocytes.[23] As circulating granulocytes of tumor patients have impaired cytotoxic activity,[24] which is further decreased by most chemotherapeutic agents,[25] the elaboration of systems capable of guiding the recruitment of PMNs and their activation within a tumor microenvironment can be put forward as a fresh therapeutic approach.

PMN recruitment into the tumor

Extravasation from the blood into a tumor is a regulated multistep process involving a series of coordinated interactions between PMNs and endothelial cells.[26] Several molecular regulator families, namely selectins, integrins, and cytokines, are thought to control the steps of this process. It is well known that P-, E-, and L-selectin adhesion molecules (also known as GMP 140, ELAM-1, and LAM-1, respectively) initially tether free-flowing neutrophils to the endothelium of postcapillary venules and mediate transient interactions by causing them to roll much more slowly. Slowly progressing PMNs pick up the signals delivered by interleukins, chemokines, and other mediators released by the endothelium and become firmly attached to it via β_2 integrin (MAC-1, ie, CD11b/CD18)–intercellular adhesion molecule-1 (ICAM-1) recognition.[27] The endothelium is thus the most active controller of leukocyte traffic and behavior through its display of specific signals.

Some proinflammatory mediators or other factors directly secreted by engineered tumor cells, or elicited as downstream

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mediators by the released cytokine, increase the endothelial expression of several leukocyte adhesion and activation molecules. IL-1β and TNF-α induce and/or up-regulate ELAM-1, P-selectin, ICAM-1, and vascular cell adhesion molecule-1 expression in endothelial cells, whereas IFN-γ mainly promotes ICAM-1 expression.[28-31] This cytokine-endothelium cross-talk is thus the first step in regulating PMN intratumoral accumulation (Figure 1).

PMN intratumoral accumulation is also attained when IL-10, a cytokine typically regarded as an anti-inflammatory mediator because it inhibits the release of other interleukins and chemokines,[32-34] is present in the microenvironment. We have demonstrated that local release of high levels of IL-10 by IL-10 gene transfected mammary carcinoma cells (TSA–IL-10) in a syngeneic host results in both an anti- and a pro-inflammatory activity, through strong endothelial ELAM-1 expression in peripheral tumor microvessels.[35] This expression and subsequent intratumoral accumulation of PMNs were directly attributable to IL-10, since no secondary mediators were detected.[35-36] Moreover, it is likely that IL-10 attenuates the constitutive endothelial cell release of nitric oxide[37] and contributes to the adhesion of PMNs to microvessels[38,39] and their intratumoral accumulation.

It has been recently reported that IL-10 up-regulates the expression of the liver-expressed chemokine (LEC), a human β chemokine also known as NCC-4, HCC-4, and LMC.[40] LEC is chemotactic for human monocytes and dendritic cells, but not for neutrophils.[41,42] Nevertheless, local accumulation of LEC secreted by engineered TSA cells (TSA-LEC) quickly induces their rejection in association with an impressive recruitment of antigen-presenting cells, lymphocytes, and particularly PMNs.[21]

PMNs intratumorally recruited by highly expressed endothelial adhesion molecules also play a key role in mounting a strong antitumor response.[21]

Induction of ELAM-1 and up-regulation of ICAM-1 in the blood vessels, even in the case of tumors formed by cells engineered to release G-CSF, IL-2, IL-4, and IL-12, are attributable to both the cytokine-released and downstream-induced secondary mediators, such as CXC chemokines.[43,44] Experiments with tumor cells transfected to release G-CSF, IL-2, and IL-12 as well as TNF-α,[45-47] have disclosed an evident production of macrophage inflammatory protein-2 (MIP-2), also known as growth-related oncogene/cytokine–induced neutrophil chemoattractant (GRO/KC).[48-50] This molecule is the murine functional homologue of human IL-8, which up-regulates the binding affinity of a family of adhesion molecules called integrins on PMNs and of its counterreceptor, ICAM-1, on endothelial cells.[51] Integrin-mediated adhesion leads to extravasation of PMNs highly attracted to the tumor site by MIP-2, which binds to the PMNs' CXCR1 or CXCR2 counterreceptor (see review in Luster[52]). MIP-2, together with other factors belonging to the IL-8 granulocyte chemoattractant family, is often released by tumor-associated macrophages under the stimulus of the cytokines released by tumor cells or the secondary mediators they induce.[53] We found that macrophages and endothelial cells were clearly stained by anti–MIP-2 antibody when TNF-α as well as IL-1β were present in the tumor microenvironment.[44,46] In all these cases, MIP-2 expression was associated with marked recruitment of PMNs, whose accumulation was further enhanced by the further release of MIP-2 produced by PMNs themselves in response to stimulation by TNF-α in the tumor microenvironment.

PMN-induced tumor destruction

Recruited PMNs produce several cytotoxic mediators, including reactive oxygen species, proteases, membrane-perforating agents, and soluble mediators of cell killing, such as TNF-α, IL-1β, and IFNs (Figure 1).

Oxidants employ 2 mechanisms to injure tumor cells. They act synergically with protease and other agents, and inactivate plasma antiproteases to allow proteases to operate.[54]

Recent dissection of the cytolytic armamentarium of PMNs has suggested a primary role for hypochlorous acid (HOCl) in mediating tumor cell lysis by activated PMNs after their leukocyte function–associated antigen 1–dependent recognition of the target cell surface.[55] Furthermore, a distinct adhesion pathway, mediated by CD11b/CD18 up-regulation on activated PMNs, enables these

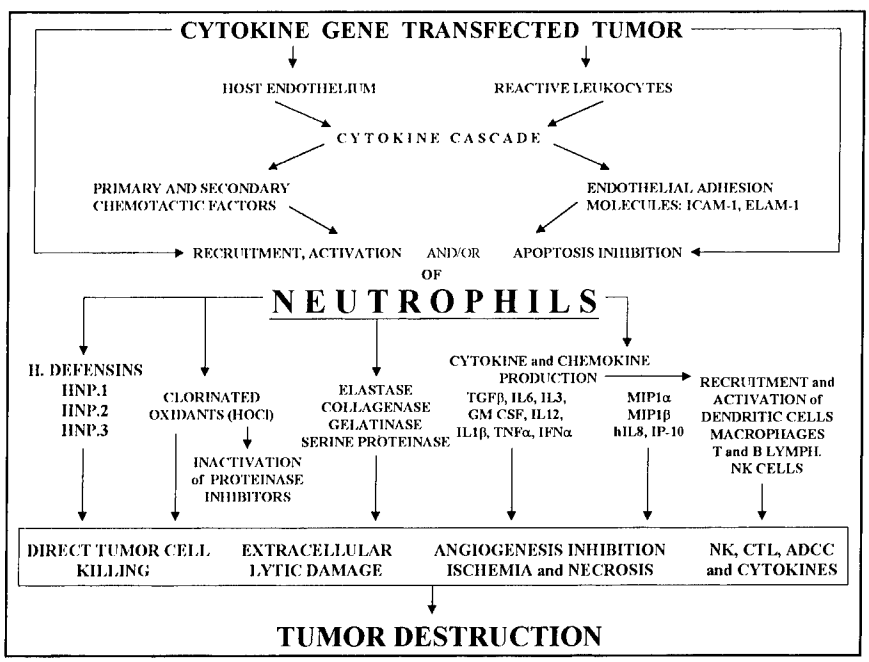

Figure 1. Recruitment of neutrophils into cytokine-transfected tumor and their involvement in its destruction. Local release of the cytokine from transfected tumor cells acts on the host endothelium and reactive leukocytes, mainly tumor-associated macrophages. The cytokine and chemokine cascade and endothelial adhesion molecule expression thus induced results in PMN recruitment and activation. Tumor destruction on the part of activated PMNs is achieved through their release of a variety of factors (cytokines, enzymes, chlorinated oxidants, etc) whose effects include direct tumor killing, extracellular lysis, inhibition of angiogenesis and activation of other reactive cells, resulting in NK cell, T cell, and antibody-dependent cytotoxicity.

cells to adhere to the vascular endothelium and create a subjacent microenvironment, allowing accumulation of oxidants and proteolytic enzymes at local concentrations sufficient to cause endothelial damage and matrix degradation.[56] In addition, PMN-released HOCl reacts with primary amines to form relatively stable chloramines with immunostimulatory properties.[57]

Although reactive oxygen and reactive nitrogen intermediates are toxic molecules that contribute to the control of tumors, they also mediate inhibition of T-cell proliferation by suppressing macrophage functions. This mechanism accounts, at least in part, for the immunosuppressed state seen in certain infectious diseases, malignancies, and graft-versus-host reactions (see review in Bogdan et al[58]).

A further mechanism of PMN-mediated tumor cell killing is antibody-dependent cell-mediated cytotoxicity (ADCC).[59] The role of antibody-independent recognition of tumor cells by cytotoxic T cells has been extensively researched, whereas only a few recent works have shown that in vivo tumor ADCC also exists.[60-63] Granulocyte-macrophage–CSF (GM-CSF) augments the normal PMN ADCC of melanoma, neuroblastoma, and colorectal cancer cells.[64-66]

Recent preclinical studies of the treatment of advanced renal cell carcinoma by combining bispecific antibodies (with one specificity against the epidermal growth factor–receptor (EGF-R) overexpressed on the majority of renal cell carcinomas, and another specificity against Fc receptors on human leukocytes) with G-CSF or GM-CSF therapy have demonstrated that granulocytes are the most active effector cell population.[67] Although systemic application of bispecific antibodies is suitable at present only for adjuvant treatment of minimal residual disease due to poor tumor cell accessibility, local administration, either alone or in combination with autologous effector cells, is highly effective in eradicating tumor cells.[68]

In our murine model, IL-10–releasing TSA cells initially grow and are then rejected by the combined action of $CD8^+$ lymphocytes, natural killer (NK) cells, and PMNs.[35,69] As already mentioned, the marked anti-TSA antibody response that follows this rejection may be responsible for PMN-mediated tumor ADCC.

A markedly high titer of anti-TSA antibodies is also elicited during the early phases of LEC-releasing TSA tumor cell rejection. Here, too, antibodies may provide further guidance for the PMN-dependent tumor rejection.[21]

A family of antimicrobicidal peptides called defensins has been described in humans.[70] Defensins are the most abundant component of the azurophil granules of PMNs and are highly toxic against several types of tumor cells.[71]

These granules also contain elastase and cathepsin G, 2 proteases particularly injurious to endothelial cells.[72] It is still uncertain whether adhesion of PMNs to tumor cells is required to cause injury. However, the ultrastructural studies performed during the growth and rejection phases of several tumors engineered to release cytokines show PMNs in close contact with injured tumor cells.[23,47] An absolute need for adhesion cannot, of course, be inferred from observations of this kind.

Histological and ultrastructural investigation of tumor growth area morphology shows that the damage produced by PMNs takes 2 forms: predominantly colliquative necrosis when their cytotoxicity against tumor cells prevails[23] and predominantly ischemic and/or hemorrhagic necrosis when their main target is the vascular endothelium.[20,23,35,73,74]

Modulation of PMN behavior by cytokines

Serial immunohistological examination and polymerase chain reaction analysis after a subcutaneous challenge with TSA–IL-2, TSA–IL-4, TSA–IL-10, TSA–IL-12, and TSA–TNF-α cells have shown that the local release of all these cytokines elicits a quick and effective PMN-mediated antitumor activity.[20,44] The kinetics, however, are not the same, and the boosted PMN functions are often different.

Presence of pro-inflammatory cytokines

Paracrine release of IL-2, IL-4, IL-12, and TNF-α induced prompt tumor rejection (Figure 2A) marked by the formation of areas of colliquative necrosis typically associated with a massive presence of degranulated PMNs with exocyted granules in close contact with tumor cells displaying ultrastructural signs of irreversible damage (Figure 2B). This indication of direct killing by PMNs was particularly evident in the presence of paracrine IL-2 and TNF-α.

Interestingly, the observed release of TNF-α into the circulation during infusional recombinant (r) IL-2 therapy of cancer patients is often associated with a potent activation of PMNs that interact with tumor cells and endothelial cells and cause their subsequent lysis.[75,76] According to this killing activity, PMNs appear to mediate most of the therapeutic efficacy, but also the systemic toxicity of rIL-2 (ie, vascular leak syndrome).[77] However, these 2 opposite effects might converge in a single antitumor effect with the development of intratumoral cytokine gene transfer therapy.

In mice, direct killing by PMNs was also a hallmark of the rejection of tumor cells engineered to release G-CSF. Activated PMNs with prominent cytoplasmic projections, in fact, were observed in close contact with dead tumor cells[47,78] well before any vascularization of injected tumors was possible. When recipient

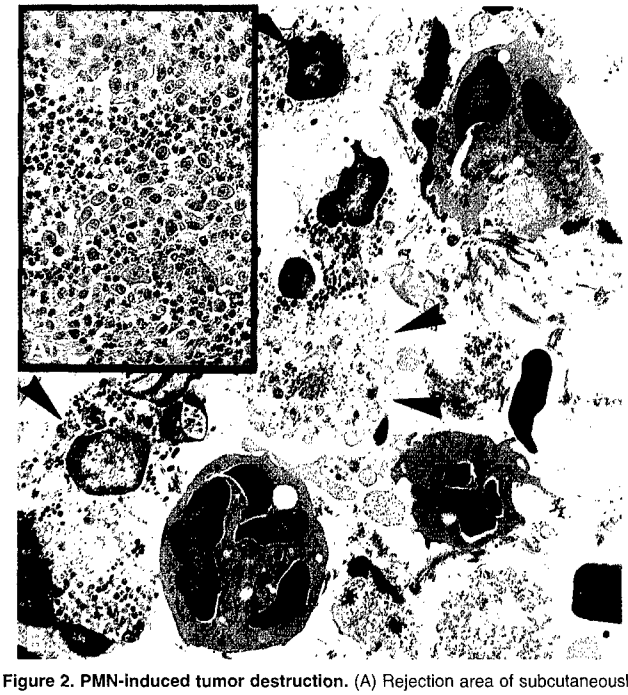

Figure 2. PMN-induced tumor destruction. (A) Rejection area of subcutaneously injected tumor cells engineered to release IL-2 is massively infiltrated by polymorphonuclear leukocytes (× 630). (B) Electron micrograph showing that these are neutrophils at various stages of disorganization and that their exocytosed granules are in close contact with severely damaged or necrotic tumor cells (× 2750).

mice were sublethally irradiated, tumors grew and became vascularized before the rejection that occurred when PMNs and leukocytes were self-reconstituted. In this case, the tumor-associated blood vessels were the main PMN target.[78]

Immunohistological analysis showed that in all these situations IL-1β and TNF-α were the predominant downstream pro-inflammatory cytokines elicited in the tumor growth area. They were usually associated with MIP-2 expression in both tumor-associated macrophages and PMNs. Indeed, by thus inducing macrophages to produce PMN chemoattractants, they set up a vicious circle, since they also stimulate PMNs to produce MIP-2 and probably other factors belonging to the IL-8 granulocyte chemoattractant family (Figure 3).

The marked and rapid PMN influx and tumor necrosis observed in nude mice challenged with human tumor cells engineered to release IL-8 (the functional human equivalent of MIP-2), human MIP-1α, and murine MIP-1α[79] show that both CXC chemokines, such as IL-8 and GROs, and specific CC chemokines, such as MIP-1α, regulate PMN traffic and functions, providing further evidence that recruited PMNs suppress tumor growth.

Absence of pro-inflammatory cytokines

Rejection of TSA–IL-10 cells, on the other hand, is paradigmatically different.

By suppressing the release of pro-inflammatory cytokines such as IL-1β, TNF-α, and GM-CSF, paracrine IL-10 impairs the early influx of PMNs and permits initial tumor formation by transiently paralyzing a prompt nonspecific antitumor response.[35,69] However, by directly acting on endothelial cells, it induces adhesion molecules and attenuates their nitric oxide release, thus favoring leukocyte recruitment in vivo.[36,80,81] Leukocytes and mainly neutrophils are responsible for the intratumoral microvasculature damage that results in multiple ischemic necrotic areas.

Local necrosis is followed by massive infiltration by PMNs and a few T and NK cells, probably recruited by mediators induced by hypoxia and necrosis.[82] The late influx of reactive cells, however, is enough to lead to complete rejection of the established tumor. This does not take place in the absence of PMNs.[69]

The well-known immunosuppressive activity demonstrated by IL-10 in vitro has naturally impeded assessment of its potential use in the treatment of solid human tumors. Its employment has been proposed only for management of the rare myeloproliferative disorder called juvenile myelomonocytic leukemia.[83] Even here, consideration has been devoted solely to its ability to inhibit the production of cytokines and growth factors by myelomonocytes in vitro.[83] Animal data, however, suggest that intratumoral administration of IL-10 inhibits tumor growth through a pro-inflammatory activity in which PMNs again play a fundamental role.[35,69]

Presence of IFN-γ

The presence in the tumor growth area of appreciable amounts of IFN-γ together with IL-1β and TNF-α was noted after injection of TSA cells engineered to release IL-2, IL-4, and IL-12.[43,44] It was even more evident when local or systemic administration of rIL-12 in mice bearing a subcutaneous 7-day-old TSA tumor resulted in intratumoral expression of the messenger RNA of IL-1β, TNF-α, and IFN-γ, together with IP-10 and MIG,[44] 2 chemokines with well-known antiangiogenic activities. IL-1β and TNF-α probably induced the production of PMN chemoattractants by tumor-associated macrophages, as the number of tumor-infiltrating PMNs was significantly enhanced after 3 intraperitoneal administrations of rIL-12.

The presence of IFN-γ as secondary mediator, however, must also have induced both macrophages and PMNs to produce IP-10 and MIG[84,85] (Figure 3). The rapid influx of PMNs with a high destructive potential and an IP-10– and MIG-mediated anti-angiogenic function resulted in vascular damage, inhibition of angiogenesis, and extensive ischemic/hemorrhagic necrosis after 3 and 8 rIL-12 administrations. Macrophages were undoubtedly essential modulators of this immune response. The crucial importance of PMNs, however, was made clear when their selective depletion abolished the rIL-12–induced antitumor effect. Whether PMNs also have a role in IL-12 therapy of human cancer has still to be determined.

IP-10 and MIG are also intense chemoattractants for monocytes and T cells.[86-88] They promote T-cell adhesion to endothelial cells and are leading recruiters of the T cells, particularly $CD8^+$, found to be indispensable, like PMNs, for complete eradication of most of our inocula and experimental tumors.

The presence of IFN-γ and its induction of a cytokine cascade are usually associated with an elevated antitumor memory reaction, since all inocula with IFN-γ in the growth area led to the rejection of a secondary challenge (Figure 3).

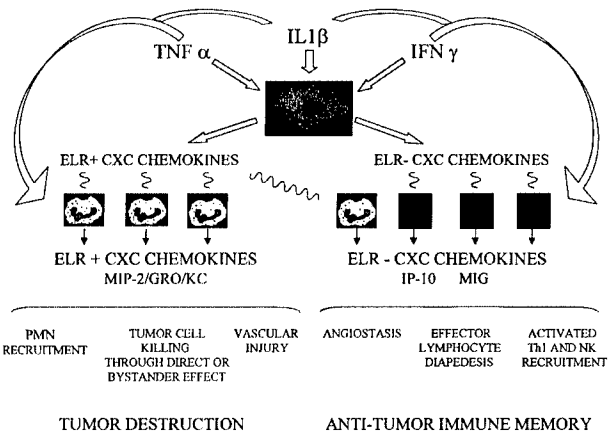

Figure 3. Cytokine modulation of PMN intratumoral recruitment and functions. Cytokine modulation of PMN intratumoral recruitment and functions results in tumor destruction and an antitumor immune memory. Tumor-associated macrophages are the first reactive cells ready to respond to the secondary mediators (IL-1β, TNF-α, IFN-γ, etc) induced by the cytokine systemically administered or locally released by engineered tumor cells. Depending on the cytokines present in the microenvironment, activated macrophages release ELR (glutamic acid–leucine–arginine)$^+$ CXC chemokines, which recruit neutrophils, and/or ELR$^-$ CXC chemokines, which recruit T lymphocytes. If IL-1β and TNF-α prevail, macrophages and PMNs are induced to produce ELR$^+$ chemokines (MIP-2/GRO/KC), which amplify accumulation of PMNs and favor their destructive functions. If IFN-γ prevails, they are induced to produce angiostatic ELR$^-$ chemokines (interferon inducible protein-10 [IP-10], monokine induced by gamma interferon [MIG]) that recruit T lymphocytes and promote the establishment of an antitumor immune memory.

PMNs and the antitumor immune memory

PMNs do not seem of great importance in the elaboration of a significant immune memory against the secondary tumor cell challenge. Even so, they are certainly one of the effector arms involved in the destruction of a second inoculum that takes place once this memory is established. This was particularly evident in the case of TSA cells transfected with the IFN-γ gene.[20,23] This tumor, in which the PMN infiltrate is almost absent, was rejected in only 25% of cases, though the immune memory established after

the first challenge always provided complete protection against a subsequent TSA–parental cell inoculum.

Provided there is IFN-γ in the tumor growth area, the cytokine released by the engineered tumor cells plays a significant role in skewing the memory reaction toward T_H1 and T_H2. However, secondary rejection is not simply the work of T cells. Massive PMN recruitment, in fact, is consistently evident in both T_H1- and T_H2-deflected memories. Since their selective removal impairs or even abolishes rejection after establishment of the immune memory,[21,69,89] it may well be that PMNs play hitherto unsuspected roles.

PMN-mediated ADCC may significantly contribute to the immune memory leading to secondary tumor cell rejection.[59,64,65] However, there may be a more complex interplay in which T cells release a guidance factor that directs the powerful destructive action of PMNs. Close contacts between granulocytes, lymphocytes, and tumor cells are typical of immune memory reactions, in keeping with the possibility that cytolytic activity of granulocytes is guided by factors secreted by lymphocytes.

PMNs and anticancer therapy in humans

New, interesting perspectives are opening to exploit PMN functions for anticancer strategies in patients.

During therapy with G-CSF, significantly enhanced in vitro cytotoxicity of isolated PMNs against glioblastoma, squamous cell, ovarian, and breast carcinoma cells was observed with sensitizing monoclonal antibody to the oncogene products EGF-R and HER-2/neu.[90-92]

Indeed, a phase I study in patients with breast and ovarian cancer showed that biological and clinical activities at well-tolerated doses of bispecific antibodies to FcRI selectively induced on neutrophils and HER-2/neu expressed on tumor target cells.[91,92]

In hematologic malignancies, where tumor cells are relatively accessible to antibodies and effector cells, malignant B cells displayed a particularly high susceptibility to neutrophil-mediated ADCC.[93]

Phenotypic and functional evidence of potent PMN activation has also been observed during rIL-2 infusion in patients with advanced malignant melanoma and renal cell carcinoma.[94,95] Furthermore, patients showing disease response to treatment have a significantly greater production of PMN oxidants, such as HOCl, which is regarded as instrumental in tumor cell lysis.[55,77]

Evidence suggests that production of the tumoricidal long-lived oxidant HOCl, along with up-regulation of PMN surface integrins, may also contribute to the antineoplastic efficacy of infusional therapy with TNF-α.[96-98]

However, besides their involvement in the therapeutic efficacy of certain cytokines, PMNs may be partly responsible for the toxic side effects of high-dose systemic cytokine therapy.[77]

Conclusion

The poor results obtained in humans with systemic cytokine therapy have cast a shadow over this type of approach, especially since its failures were ascribable mainly to multiple side effects and enrollment in phase I trials of patients with advanced disease and a deeply compromised immune system. The extremely encouraging results obtained with local intratumoral cytokine release in animal models, on the other hand, suggest the possibility of successful antitumor management through an improvement in cytokine gene therapy biotechnology and procedures for patients with a reasonable immunological performance and low tumor load or minimal residual disease.

It is clear, in any event, that in addition to being the body's main defenders against infection and foreign invaders, PMNs could be a perfect weapon for the suppression of tumor growth and tumor rejection in T-cell memory reactions, while their ability to respond to and produce cytokines[2,3] involves them in the cross-talk between tumor cells and the endothelium and between nonspecific and specific immune cells.[5]

A deeper insight into the biological role of immunoregulatory molecules acting as cytokines that stimulate specific PMN functions may thus lead to the elaboration of a new approach to the treatment of cancer.

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Perspectives in Cancer Research

Immunoprevention of Cancer: Is the Time Ripe?[1]

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Abstract

Immunotherapy applied to patients with established tumors rarely leads to an objective response, whereas patients apparently free from disease after conventional treatment and at risk of recurrence are beginning to receive vaccination. New classes of patients or not-yet patients are those with a high genetic or environmental risk of developing cancer. They may draw benefit from a "soft" treatment such as vaccination. This overview discusses the prospects of immune stimulation as a means of cancer prevention by inducing various forms of nonspecific or even specific immunity. Attainment of this goal provides the rationale and motivation for embarking on such a new and potentially rewarding enterprise.

Immunotherapy is emerging as an effective way to cure cancer (1–4) thanks to the dramatic progress that has led to the molecular and genetic definition of the tumor-host immune relationship. A detailed characterization of many tumor cell surface molecules that act as TAAs[3] is now available (5, 6). A second cornerstone has been provided by elucidation of the way in which TAA peptides are presented to T lymphocytes in association with glycoproteins of the MHC (7, 8) and the role of dendritic cells (9) and costimulatory (10), danger (11), and cytokine (12) signals. Genetic engineering of antibody molecules (13), soluble costimulatory signals (14–16), and tumor (17) and dendritic (18) cells is used to intensify the immune response and skew it toward Th1 or Th2 reactivity. This crucial information forms the groundwork for most ongoing immunotherapy clinical trials whose clinical setting is elicitation of an immune response in a tumor-bearing patient.

Determination of which kind of patients are eligible for Phase III clinical trials is not a minor issue (19, 20). Practical and ethical constraints result in the enrollment of advanced cancer patients in Phase I trials, whereas experimental mouse data suggest that the immunity induced by specific vaccination is much more effective in the inhibition of incipient tumors than in the cure of established tumors. Elicitation of a significant response in animals with advanced tumors is exceedingly difficult, and only a minority of tumor-bearing mice are cured (21). As a tumor increases in size, it becomes refractory to immunotherapy. Its genetic instability leads to the selection of antigenic variant clones (22, 23), whereas the characteristics of its stroma (24), the peculiarity of its blood vessels (25), and its release of immunosuppressive factors (26) build up a sort of privileged site proof against immune attack.

A similar picture is emerging from Phase I immunotherapy trials. Only a few patients with established tumors display objective and in any event temporary responses (2, 3). The immunological performance status of the patients enrolled is obviously suboptimal. Most have already been treated in various ways and no longer respond to conventional therapy. Their tumor cells are selected because of their ability to escape immune reactions, and their tumor masses can suppress an immune attack. At present, immunotherapy seeks to overcome these obstacles by aggressive or combined forms of treatment (21), whereas it is becoming evident that active immunotherapy is probably not a rational option in advanced cases. Indeed, repeated failures could even jeopardize the whole of what immunotherapy is endeavoring to achieve.

However, the lethality of a tumor usually stems from the relatively small number of its cells that remain after its surgical excision and are not killed by radiotherapy and chemotherapy. The importance of this issue lies in the experimental demonstration that active immunotherapy is effective against minimum residual disease and incipient metastases and in the control of tumor recurrences (27). Early immunotherapy after a successful conventional treatment is warranted. Clinical trials suggest that patients with minimal residual disease or patients expected to present recurrences after a long interval are those for whom immunotherapy may prove really effective because it induces a prolonged tumor free-survival, if not a complete cure (1, 4). Cancer vaccines tested as single agents in advanced melanoma patients are being tested in apparently disease-free patients in combination with chemotherapy. Significant results are expected from this more rational clinical approach (28). Once the efficacy of therapeutic immunization is demonstrated, it may also be proposed as an at-home or outpatient method for the elicitation of a long-lasting immunity after the conventional management of a small tumor (17).

The Prospects of Prevention

If immunotherapy is most effective in the early stages of tumor growth, consideration can be given to an even more radical view. The use of immunological measures to prevent or forestall cancer in healthy persons has not received much attention. This is surprising because most of the experimental data obtained with transplantable tumors show that new vaccines preimmunize mice against even a poorly or apparently nonimmunogenic tumor challenge (12, 29). Furthermore, the nonspecific immunity elicited by local and systemic cytokines effectively inhibits incipient tumors until they overcome a critical threshold and become clinically evident (27). Numerous and unambiguous experimental data show that the efficacy of both nonspecific and specific immunity declines as a tumor progresses (21, 27). Whether willfully or unthinkingly, however, the evidence from preimmunization tumor challenge experiments and the cytokine-induced collapse of incipient tumors is strained to demonstrate the efficacy of immunological measures in tumor therapy and not accepted for what it really says (30), namely, that immune reactivity possesses a great preventive potential, whereas its real therapeutic efficacy against established tumors is altogether another question (17).

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[3] The abbreviations used are: TAA, tumor-associated antigen; IL, interleukin; neuT, transforming HER-2/*neu* oncogene; neuN, nonmutated HER-2/*neu* proto-oncogene; Th, T helper.

Appendix 9

Immunoprevention of cancer would have many advantages on its side. Healthy subjects, for example, may be expected to mount a more effective and powerful response than patients who have already been treated in various ways, whereas if the target tissue is still normal or displays no more than a localized preneoplastic lesion, the chances of success should be greater than when dealing with unresectable or disseminated tumors (31). Preneoplastic lesions do not yet display genetic instability, TAA mutations, and selection of the TAA-negative clones that characterize established tumors. They should also be more permeable to immune mechanisms because their cells do not markedly remodel the extracellular matrix or produce suppressive factors, and their vessel endothelium is not yet refractory to leukocyte extravasation (32–34). Several mutations in oncogene products are required for transformation. By contrast, an alerted immune patrol would detect the initial mutations and be ready to intervene before complete transformation takes place. Although antigen(s) associated with preneoplastic lesions (as well as those for many established neoplasms) have not been yet identified, the products of mutated oncogenes are probable candidates (35). Moreover, papillomas induced by methylcholanthrene are both antigenic and antigenically distinct from each other. This suggests that TAAs characterizing subsequent progressing sarcomas are already present at the preneoplastic stage (36).

Nonspecific Immunity in Cancer Prevention

The selection of not-yet patients and healthy individuals eligible for immunoprevention depends on the kind of treatment envisaged. Enhancement of nonspecific immunity and specific antitumor vaccination are two possible approaches. The advantage of a nonspecific antitumor response is that it can be applied directly to a broad range of individuals, irrespective of the type of TAA their foreseeable tumors may eventually express. However, it is not feasible to imagine healthy persons being treated nonspecifically for long periods. The results of the mouse experiments indicate that nonspecific stimulation should thus be restricted to not-yet patients with a genetic risk of cancer (34), individuals exposed to high carcinogen doses (37), patients with a preneoplastic lesion, and patients that probably have minimal residual disease after successful conventional treatment (27). Many not-yet patients with a high risk of cancer are, in fact, being recruited in ongoing programs to screen for preneoplastic lesions or gene mutations that predispose to cancer.

Women at risk for breast cancer or with preneoplastic lesions form a category for which nonspecific immunoprevention could be considered as a practical option.

However, the disclosure of a genetic risk of cancer and the presence of a preneoplastic lesion raise complex issues (38). Not a few individuals will find it difficult to cope with this information and may become deeply anxious about the possibility that they may have cancer. Routine cancer screenings, prophylactic mastectomy, and chemoprevention are all unpleasant and additionally stressful options (39, 40). A "soft" immunoprevention alternative would undoubtedly be welcome.

But what has nonspecific stimulation of immune reactivity to offer? A study of immunosurveillance against preneoplastic skin carcinomas suggested that it is not selective because elimination of such lesions was in no way related to their degree of malignancy (41). The extent to which nonspecific stimulation can prevent the onset of cancer in cases where a risk exists has been investigated by Noguchi *et al.* (37) in Dr. L. J. Old's laboratory. In their experiments, tumors were induced in BALB/c mice by s.c. injection of 3-methylcholanthrene. Delayed tumor appearance and reduced incidence were observed in mice receiving 100 ng of systemic IL-12 five days a week for 18 weeks (3 weeks on and 1 week off) during tumor latency. Secondary IFN-γ, IL-10, and tumor necrosis factor-α were evident in their sera. A high production of IFN-γ by CD8 T cells and a Th2$\rightarrow$Th1 or Th0 shift in the cytokine secretion profile of CD4 T cells were also noted.

The ability of similar doses of IL-12 to prevent tumors when administered to mice with a genetic risk of cancer was therefore studied by us (34) in two lines of transgenic mice expressing the rat HER-2/*neu* oncogene under the transcriptional control of mouse mammary tumor virus (34). Female BALB-neuT (H-2^d) mice carrying the transforming HER-2/*neu* oncogene show no morphological abnormalities of the mammary gland until 3 weeks of age. They then progress through atypical hyperplasia to *in situ* lobular carcinoma, and at 33 weeks of age, all 10 mammary glands display invasive carcinomas. In adult FVB-neuN (H-2^q) mice carrying the HER-2/*neu* proto-oncogene, neoplastic progression is less impetuous, as shown by a longer latency (38–49 weeks) and a lower tumor multiplicity (mean, 2.6 tumors/mouse). Treatment with IL-12 (five daily i.p. injections; 1 week on and 3 weeks off; the first course with 50 ng IL-12/day and the following courses with 100 ng IL-12/day) begun at 2 weeks of age in BALB-neuT mice and at the 21 weeks of age in FVB-neuN mice markedly delayed tumor onset and reduced tumor multiplicity. In both lines, tumor inhibition was associated with deficient peri- and intratumoral angiogenesis, infiltration of reactive cells, production of proinflammatory cytokines, and inducible nitric oxide synthetase activation.

We next set out to determine the stage at which administration of IL-12 is most effective. Was it simply a preventive measure in still healthy animals or could it also be of benefit once overt preneoplastic lesions are diagnosed? Groups of BALB-neuT and FVB-neuN mice received IL-12 at progressive times during carcinogenesis (42). In both lines, IL-12 was particularly effective in inhibiting the progression from hyperplasia to *in situ* and invasive carcinoma, *i.e.*, at the time of the angiogenic switch. Its antiangiogenic effect is markedly evident on the fragile capillaries sprouted during this switch. Late administration was poorly effective in both mouse lines, presumably because the mature and differentiated blood vessels of more advanced lesions are less sensitive to IL-12-induced inhibition. However, the antitumor action of IL-12 is not confined to its indirect influence on endothelial cells. Directly or through secondary cytokines, it triggers lytic activity and mediator release from a variety of tumor-infiltrating leukocytes and thus counters the continuous generation of transformed cells. Its efficacy, in fact, probably rests on the sum of its activities, and not simply on the blocking of tumor angiogenesis, important as this may well be (27). These experiments also show that lifelong administration is not required for genetically determined cancers with a long natural history. Precise definition of the carcinogenic events may allow preventive treatments to be performed only during a critical stage of the long carcinogenic progression.

The HER-2/*neu* oncogene is expressed in a substantial proportion of human mammary carcinomas. The close resemblance of the progression of mammary carcinogenesis in HER-2/*neu* transgenic mice to that in women suggests that the administration of nontoxic recombinant IL-12 regimens may be a significant prophylactic strategy. The direct proportionality between the length of carcinogenesis progression and the efficacy of IL-12 observed in these models suggests that stimulation of nonspecific immunity could be envisaged as an effective, preventive way of slowing human carcinomas (30).

The principles illustrated by these models are clear. The extent to which they reflect the situation in humans must obviously be established in clinical trials, especially because the immunological weight of IL-12 may not be the same in mouse and human tumors. In the meantime, further evidence that cytokine-elicited immunity can prevent tumor progression is provided by a randomized multicenter Phase III trial with low, nontoxic doses of IL-2 injected locally.

Patients with resectable $T_{2-4}N_{0-3}M$ squamous cell carcinomas of the head and neck receiving supplemental IL-2 before and after surgery displayed a significantly extended disease-free interval as compared with those treated only with conventional therapy.[4]

Specific Antitumor Vaccination of Persons at High Risk of Cancer

Specific vaccination of persons at risk and healthy individuals constitutes a very different scenario. Characterization of specific gene alterations or detection of preneoplastic lesions may indicate which organ and tissue are at risk. In a few cases, more precise information may show which oncogene product will probably be overexpressed or expressed in an altered form and allow vaccination against a single, specific TAA. Molecular characterization of altered gene products predictably destined to become TAAs will be the first step toward the engineering of selective vaccines (43). Otherwise, the patient should be vaccinated against the TAA most commonly expressed by the tumors foreseeable in a given organ.

Many new antitumor vaccines that induce an effective resistance to subsequent tumor challenge and inhibit minimal residual disease are already available (12, 29). The question of whether specific immunization can be successful once a cell population has been subjected to the initial carcinogenic hit has rarely been examined experimentally. However, it can be plausibly suggested that cytokines and more conventional adjuvants could induce an effective immune response against ignored or fully tolerated antigens. The specific immunity elicited in mice transgenic for rat Her-2/*neu* is a sign that specific vaccination induces strong immune responses against such antigens and may thus inhibit oncogenesis and extend survival (44–46).

General Antitumor Vaccination

One can also envisage the even wider application of antitumor vaccines to prevent tumors in the general population, as is done for infectious diseases. This point considers the possibility of preventing the onset of cancers related to an infectious agent by vaccination against the agent itself. This approach is applicable to a sizeable proportion of diverse human tumors including cervical carcinoma (human papillomaviruses), hepatocellular carcinoma (hepatitis B and C viruses), and Burkitt's lymphoma (EBV). A significant impact of hepatitis B vaccination on the incidence of hepatocellular carcinoma has already been reported (47), and promising results being obtained in preclinical models of papillomavirus oncogenesis (48) suggest that human vaccination will eventually be able to prevent cervical carcinoma (49).

Molecular and genetic data suggest that human TAAs identified as targets of CTLs (5) or by the SEREX technique (6) can be divided into classes. One class consists of tumor-specific antigens coded by genes expressed by tumors but not by normal cells, with the exception of male germinal cells. However, because these cells do not express MHC glycoproteins, they do not present peptides from the protein products of these genes on their surface. The use of these antigens in preventive vaccination is interesting because their number seems not to be endless, and they are shared by histologically distinct tumors arising in different organs. Furthermore, the telomerase catalytic subunit is markedly activated in more than 85% of human tumors, whereas it is silent in normal tissues and thus constitutes a sort of universal TAA (50). The second class of antigens derived from point mutations looks less interesting for general vaccination because they are unique for a given tumor, and their expression by a foreseeable tumor is poorly predictable. Nevertheless, in some cases, oncogenes and oncosuppressor genes display a narrow spectrum of mutations (*e.g.*, RAS; Ref. 51). In addition, chromosome translocations that give rise to fusion proteins display a relatively constant pattern of junction between the two genes.

Another class comprises antigens that are also expressed by normal cells of the same differentiation lineage. Immune reactions elicited against them could be coupled with the induction of an autoimmune disease. An additional class is formed of molecules expressed by normal cells and overexpressed by neoplastic cells. Here, too, there is a risk of inducing autoimmune reactions. In fact, once the immunological ignorance or tolerance against these antigens is overcome, effector mechanisms endowed with a lower threshold of activation may destroy both normal and neoplastic cells. However, experimental data from variously immunized mice did not disclose major autoimmune lesions as a side effect of vaccination with these antigens. On the contrary, a specific immune reaction often affected tumor cells overexpressing the target TAA and spared normal tissues where TAA was expressed at a much lower level (52).

Because many TAAs are shared by a variety of tumors, preventive immunization against most common human cancers with not many more than 20 TAAs would seem conceivable. A possible list would include the infectious agents mentioned earlier, mutated oncogenes, telomerase catalytic subunit, and antigen of the MAGE family. However, the erratic boundary between tumor immunity and autoimmunity (53) means that the risk of inducing an autoimmune disease is a major concern. This risk would be much weightier in the vaccination of healthy individuals as opposed to individuals at risk, where the scales of risk-benefit are markedly biased by the higher risk of cancer and the consequent shorter life expectancy. A further warning is related to "epitope spreading" (54). Several data in animal models show that immune responses to a few self-determinants shift drift and diversify with time and include other epitopes of the same proteins or other proteins.

The planning of vaccines à la carte by genetic engineering may be a way to selectively trigger reaction mechanisms that ignore cells that express a low density of the target antigens or are less prone to induce a widespread autoimmunity. Consideration must also be given to the balance between the kind of potential autoimmunity and the degree of lethality of the possible tumor. Autoimmune vitiligo, for example, would be a relatively small price to pay for protection against melanoma, whereas in other situations, such as the prevention of bowel tumors, the risk of more severe autoimmune diseases would demand a careful approach. Experimental studies should address this issue in detail.

Another limitation to be carefully weighed is the constraint imposed by the polymorphism of MHC glycoproteins and the repertoire of peptides presented. Different peptides would need to be prepared to fit in the polymorphic peptide-binding clefts of the many MHC class I and II glycoproteins. It is predictable that certain TAAs will have a restricted usage, and a few individuals will not be easily vaccinated.

Elicitation of a "surgical" immune response ablating only cells that express a specific antigen is probably impossible. This does not mean, however, that individualized vaccines are a strict necessity. Vaccines have to be reprocessed by the immune system of the host. Therefore, in many instances, the presence of inappropriate antigens, for example, allogeneic MHC molecules, could result in the establishment of a polyclonal T-cell activator that would favor and not hamper the induction of a restricted, peptide-specific immune response (43, 44, 55).

[4] A. De Stefani, G. Forni, R. Ragona, G. P. Cavallo, M. Bussi, A. Usai, F. Badellino, and G. Cortesina. Improved survival with perilymphatic IL-2 in resectable squamous cell carcinomas of the oral cavity and oropharynx, submitted for publication.

Conclusions

The cardinal prerequisite of preventive medicine is the Hippocratic "do no harm": *"primum non nocere"* (56). Prolonged nonspecific and specific immune stimulation of persons at risk and the general population is indeed not free from uncertainty, although identification of the steps of tumor progression most susceptible to the immune mechanisms elicited could drastically reduce the stimulation period (42).

However, as stressed in a recent report on cancer chemoprevention (56), failure to intervene when a disease as diffuse and dramatic as cancer can be prevented can also be viewed as harmful. The idea that it is not appropriate to treat healthy persons with cytokines or with antitumor vaccines because of the risks involved will hopefully be shown to be a misconception. An equal or even higher risk of inducing autoimmune complications is associated with many antimicrobial vaccines. Fortunately, they came into use before this risk was perceived. Had it been otherwise, their employment would have been much more strongly opposed, and many more persons would have died.

In conclusion, immunoprevention of cancer seems a distant but plausible prospect. Experimental elucidation of its critical issues could provide essential information for its application in humans. Prevention itself would provide a fresh and perhaps conclusive way of winning the long-lasting war against cancer. Manipulation of the immune response to prevent cancer could soon lead to the realization of a notion that has deep roots in the history of immunology (57).

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Interleukin 12-activated Lymphocytes Influence Tumor Genetic Programs[1]

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ABSTRACT

T-lymphocytes (LYs) from normal and IFN-γ knockout mice were activated by anti-CD3 and anti-CD28 antibodies and cultured in inserts in the presence of interleukin (IL)-12 (IL-12-activated LYs) or not (activated LYs). Their ability to modulate the genetic programs of two tumor lines growing at the bottom of transwells was evaluated. cDNA gene expression array, reverse transcription-PCR, and protein expression showed that LPS, transcription termination factor 1, transforming growth factor, and fibroblast growth factor genes were up-modulated by factors other than IFN-γ released by activated LYs. The high levels of IFN-γ released by normal IL-12-activated LYs up-modulated the expression of STAT1, IRF-1, LMP2, LMP7, monokine induced by IFN-γ, monocyte chemoattractant protein 1, and angiopoietin 2 genes but down-modulated the expression of vascular endothelial growth factor. PA28, IFN-inducible protein 10, inducible NO synthetase, and macrophage-inhibitory protein 2 genes were up-modulated by factors released only by IL-12-activated LYs apart from IFN-γ. The opposite modulations of vascular endothelial growth factor expression and of angiopoietin 2, monokine induced by IFN-γ, IFN-inducible protein 10, and inducible NO synthetase by IL-12-activated LYs fit in well with the inhibition of angiogenesis that characterizes the antitumor activity of IL-12. T-LYs thus modify a tumor's behavior so that it becomes a party to its own inhibition.

INTRODUCTION

Cancer results from the accumulation of a series of genetic events that convert a normal cell into a transformed cell able to proliferate without restraint, attract its own blood vessels, spread around the body, and evade immune reactivity (1, 2). Numerous ways by which tumors sneak through immune reactions have been elucidated. In many cases, cells release factors that favor tumor growth by inhibiting gene expression in T-LYs[4] (3), impairing antigen presentation (4) or activating suppressor functions in macrophages (5). On the other hand, the immune system hampers tumor growth through the activity of CTLs (6), natural killer cells (7), macrophages (5), and granulocytes (8) that specifically and nonspecifically kill tumor cells. Lymphoid helper cells also may activate and guide an inflammatory-like reaction at the tumor site (9) by releasing cytokines and other factors.

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[4] The abbreviations used are: LY, lymphocyte; IL, interleukin; LPSR, LPS receptor; TNF, tumor necrosis factor; GKO, IFN-γ knockout; GM-CSF, granulocyte macrophage colony-stimulating factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; MCP1, monocyte chemoattractant protein 1; Ang, angiopoietin; MIP, macrophage inhibitory protein; IP, IFN-inducible protein; MIG, monokine induced by IFN-γ; RT, reverse transcription; mAb, monoclonal antibody; iNOS, inducible NO synthetase; STAT, signal transducers and activators of transcription; Spc, spleen cell; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IRF, interferon regulatory factor.

Exogenous proinflammatory cytokines also trigger the immune system to develop strong antitumor reactions. Systemic (10) and local (11) administration of IL-12 is particularly effective because it activates a complex reaction that halts the growth of a large number of transplantable (10, 11) as well as chemically (12) and oncogene-induced (13) primary mouse tumors. Tumor inhibition appears to result from the activation of endothelial cells and their ability to recruit lymphoid cells to the tumor site. IL-12-activated lymphoid cells kill tumor cells, damage tumor vessels, release secondary messengers, such as IFN-γ and TNF-α, and trigger the release of third-level chemokines, such as IP-10, MIG, and other factors, that counteract tumor angiogenesis (11, 14).

Here we show that by directly influencing tumor cells through the cytokines they release, lymphoid cells activated in the presence of IL-12 also modulate the growth of a tumor in a more subtle way by changing its genetic programs, so that it becomes a party to its own inhibition.

MATERIALS AND METHODS

Mice. Seven-week-old female BALB/cAnCr mice and BALB/c-GKO mice from Charles River Laboratories (Calco, Italy) were treated in accordance with Italian and European Union guidelines.

Tumor Cell Lines and *in Vitro* Cell Cultures. TSA is an aggressive and poorly immunogenic cell line established from the first *in vivo* transplant of a moderately differentiated mammary adenocarcinoma that arose spontaneously in a BALB/c female mouse (15). TUBO cells are a p185neu$^+$ cell line established from a lobular carcinoma that spontaneously arose in a BALB/c female mouse transgenic for the transforming rat Her-2/*neu* oncogene driven by the mouse mammary tumor virus promoter (13). TSA and TUBO cells express MHC class I but not MHC class II glycoproteins and spontaneously secrete granulocyte colony-stimulating factor, GM-CSF, TGF-β1, VEGF, and basic FGF (11). TSA cells were maintained in RPMI 1640 (Bio*Whittaker Europe, Verviers, Belgium) with 50 μg/ml gentamicin (Bio*Whittaker Europe), 2.5×10^{-5} M 2β-mercaptoethanol (Flow Laboratories, Opera, Italy), and 10% FCS (Life Technologies, Inc., San Giuliano Milanese, Italy; RPMI complete medium). TUBO cells were maintained in DMEM (Bio*Whittaker Europe) supplemented with 50 μg/ml gentamicin (Bio*Whittaker Europe), 2.5×10^{-5} M 2β-mercaptoethanol (Flow Laboratories), and 20% FCS (Life Technologies, Inc.; DMEM complete medium). All tumor and lymphoid cell cultures were performed in a humidified 5% CO_2 atmosphere at 37°C.

LY Activation. Total Spcs (2×10^6)/ml from normal and GKO mice were stimulated for 18 h with 2 μg/ml anti-CD3 and anti-CD28 mAbs (PharMingen, San Diego, CA) in RPMI complete medium in the presence or absence of 10 ng/ml recombinant IL-12 (Dr. Michael Brunda; Hoffmann-La Roche, Nutley, NJ). After incubation, activated LYs were washed and placed in the transwell inserts (Falcon; Becton Dickinson Labware Europe, Milan, Italy) or used in other tests. Supernatants were collected to assess cytokine contents.

Cell Proliferation Assay. A total of 200 μl of a suspension of 2×10^6 activated LYs/ml were cultured in complete RPMI 1640 in triplicate in round-bottomed 96-well plates (Falcon) and pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Milan, Italy). After 4 h, the cells were harvested on a glass fiber filter, and [^{3}H]thymidine uptake was evaluated with a Matrix-96 beta counter (Camberra-Packard, Milan, Italy). The results were expressed as the arithmetic mean ± SD of total cpm.

Transwell Tumor LY Cultures. Tumor cells (4×10^5) from confluent monolayers were cultured at the bottom of the wells of 6-well plates (Falcon) in DMEM complete medium. After 18 h, adherent tumor cells were washed

with PBS, and 3 ml of RPMI complete medium were added to each well together with a cell culture insert of 0.4 μm pore size. The insert was filled with 3 ml of complete RPMI 1640 containing 2×10^6 Spcs/ml with or without 10 ng/ml IL-12. The plates were then incubated for 96 h.

mAb to IFN-γ. Rat monoclonal IgG (An18) neutralizing IFN-γ but not IFN-α/β was produced as described elsewhere (16). Ten μg/ml high-performance liquid chromatography-fractionated An18 mAb was added to transwell cocultures. A similar preparation of rat IgG of unknown specificity was used as the negative control (16).

Titration of Cytokines in Culture Supernatants. Supernatants from activated LYs cultured in the transwells alone or in the presence of tumor cells were tested for the presence of IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and GM-CSF using sandwich ELISA kits (PharMingen).

Intracellular Detection of Phosphorylated STAT1 and STAT3 and Flow Cytometry. Tumor cells recovered by trypsinization from the transwells after 96 h of coculture with activated LYs were stained using a standard direct immunofluorescence procedure with FITC-conjugated antimouse mAb (all from PharMingen) against: (*a*) MHC class I (anti-H-$2D^d$, clone 34-2-12; anti-H-$2K^d$, clone SF1-1.1); (*b*) MHC class II (anti-I-Ad, clone 39-10-8); and (*c*) intracellular adhesion molecule 1 (anti-CD54, clone 3E2). Cells were then suspended in PBS containing 10 μg/ml propidium iodide to gate out dead cells and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). After overnight activation, LYs were analyzed for the expression of surface markers using the following mAbs (PharMingen): (*a*) anti-CD4; (*b*) anti-CD8; (*c*) anti-CD25; (*d*) anti-CD54; (*e*) anti-CD69; (*f*) anti-CD80; (*g*) anti-CD86; (*h*) anti-CD154; (*i*) anti-H-$2D^d$; (*j*) anti H-$2K^d$; (*k*) anti I-A^d; and (*l*) anti-B220.2. For the intracellular detection of phosphorylated STAT1 and STAT3, both tumor and lymphoid cells were treated as described previously by Fleisher *et al.* (17). Briefly, after the fixation and permeabilization steps, 1×10^6 cells were incubated with 0.1 μg of rabbit IgG (Caltag Laboratories, Burlingame, CA) and 1 μg of rabbit antiphosphorylated STAT1 or rabbit antiphosphorylated STAT3 polyclonal antibodies (New England Biolabs, Beverly, MA) at 4°C for 30 min. Cells were washed twice and stained with 1 μg of FITC-conjugated $F(ab')_2$ swine antirabbit IgG (Dako, Glostrup, Denmark) and incubated for 30 min at 4°C. After a final washing step, the cells were suspended in 200 μl of PBS and analyzed with a FACScan flow cytometer (Becton Dickinson). Each plot represents the results from 10,000 events.

DNase Treatment of Total RNA. To avoid contaminations of genomic DNA, 40 μg of total RNA were incubated with 4 units of DNase I (Clontech, Palo Alto, CA) for 30 min at 37°C. The digestion was stopped with 20 μl of 10× termination mix [0.1 M EDTA (pH 8.0) and 1 mg/ml glycogen], and the RNA was extracted once with 300 μl of 2:1 (v:v) phenol/chloroform solution [equilibrated with 0.1 M sodium citrate (pH 4.5) and 1 mM EDTA] and then with 200 μl of chloroform. The aqueous phase was precipitated by adding 0.1 volume of 2 M NaOAc (pH 4.5) and 2.5 volumes of 95% ethanol (−20°C, 20 min). After centrifugation (14,000 rpm at 4°C for 15 min), the pellet was washed with 80% ethanol and air dried. RNA was dissolved in 10 μl of RNase-free water. Its concentration was determined at $A_{260\ nm}$, and its quality was evaluated by denaturing agarose gel analysis.

Probe Synthesis. RNA labeling was performed with Superscript II Reverse Transcriptase (Life Technologies, Inc., Grand Island, NY) as follows: 500 ng to 1 μg of total RNA and 1 μl of 10× CDS primer mix (Atlas cDNA Expression Arrays; Clontech) in a final volume of 10 μl were incubated at 70°C for 10 min and then chilled on ice. The primed RNA was incubated at 37°C for 90 min with 6 μl of 5× First Strand Buffer (Life Technologies, Inc.), 1 μl of 0.1 M DTT (Sigma), 1.5 μl of a deoxynucleotide triphosphate mixture (20 mM dCTP, 20 mM dGTP, and 20 mM dTTP), 1.5 μl of Superscript II Reverse Transcriptase (Life Technologies, Inc.), and 10 μl of [α-^{33}P]dATP (10 mCi/ml; 3000 Ci/mmol specific activity; Amersham International, Bucks, United Kingdom). Labeled cDNA was purified by column chromatography with the Chroma Spin 200 DEPC-H_2O columns (Atlas cDNA Expression Arrays; Clontech).

Hybridization of cDNA Probes to the Array. cDNA probes (~200 μl) were first denatured with 22 μl of 10× denaturing solution (1 M NaOH and 10 mM EDTA) for 20 min at 68°C. The probe solution was neutralized with 225 μl of 2× neutralization solution [1 M NaH_2PO_4 (pH 7.0)] and incubated at 68°C for 10 min with 5 μl of C_0t DNA (Clontech). Atlas Mouse cDNA Expression Array I (Clontech) is a positively charged nylon membrane (8 × 12 cm) spotted in duplicate with cDNA fragments representing 588 known genes and 21 housekeeping genes or control sequences. These arrays were prehybridized at 68°C for 30 min with 5 ml of ExpressHyb (Clontech) and 500 μg of heat-denatured sheared salmon testes DNA. Hybridization was performed for 18 h at 68°C in roller bottles, with continuous agitation. Arrays were washed four times in 150 ml of 2× SSC and 1% SDS and once in 150 ml of 0.1× SSC and 0.5% SDS at 68°C for 30 min with continuous agitation. One final wash in 150 ml of 2× SSC was performed at room temperature for 5 min. Hybridized arrays were exposed in a phosphorimaging cassette for 20 h.

Data Analysis. The numerical data corresponding to the integrated radioactive intensity of each DNA array spot (radioactivity volume) were generated by phosphorimager analysis. They were analyzed with the DNA_MAP program. DNA_MAP analysis approach information can be obtained on the internet.[5]

RT-PCR Analysis. Total cellular RNA was extracted from TUBO and TSA tumor cells cocultured in the transwell with activated LYs as described by Chomczynski and Sacchi (18) by using the RNAble solution (Eurobio, Les Ulis Cedex, France). cDNA was prepared by reverse transcription at 42°C for 30 min in a 50-μl reaction mixture containing 4 μg of total RNA, 0.5 μg of oligo(dT), 1 mM 2′-deoxynucleotide-5′-triphosphate, 5 μl of 10× RT buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, and 1% Triton X-100], 5 mM $MgCl_2$, 40 units of recombinant RNasin RNase inhibitor, and 25 units of avian Moloney virus reverse transcriptase. All reagents for cDNA synthesis were from Promega Corp. (Madison, WI). cDNA (2.5 μl) from each sample (generated using 0.2 μg of total RNA) was amplified using a 9600 Thermal Cycle (PE Biosystem, Norwalk, CT) in a final volume of 25 μl with individual PCR cycle conditions for each set of primers. Specific primers for mouse G3PDH and iNOS were obtained from Clontech and used in the following conditions: 1× PCR Buffer, 0.2 mM deoxynucleotide triphosphates, 1 mM $MgCl_2$, 0.2 μM each primer, and 1 unit of Taq polymerase (all from Polymed, Florence, Italy). The other primers used in this work were designed on the basis of the gene sequences: (*a*) LMP2, 5′-AGGAACAGCAGTGGTGAACC-3′ and 5′-TGTAGGAGCTTCCAGAACCG-3′ (amplified fragment of 334 bp); (*b*) LMP7, 5′-CACACTCGCCTTCAAGTTCC-3′ and 5′-AACCGTCTTCCTTCATGTGG-3′ (amplified fragment of 554 bp); (*c*) PA28, 5′-AGGAGGCTGATGACTTCCTC-3′ and 5′-TCCAGACTTCTGGCTTAACC-3′ (amplified fragment of 260 bp); (*d*) IP-10, 5′-GCGTTAACCTCCCCATCAGCACCATGAAC-3′ and 5′-CCGCTCGAGGTGGCTTCTCTCCAGTTAAGGA-3′ (amplified fragment of 300 bp); (*e*) MIG1, 5′-TCCGCTGTTCTTTTCCTTTTGG-3′ and 5′-TTGAACGACGACGACTTTGGGG-3′ (amplified fragment of 361 bp); (*f*) MCP1, 5′-GCTCTCTCTTCCTCCACCAC-3′ and 5′-CGGGTCAACTTCACATTCAA-3′ (amplified fragment of 383 bp); (*g*) VEGF, 5′-CACAGCCAATGTGAATGCA-3′ and 5′-ACGTAGATCTTCACTTTCGCGGCTTCCG-3′ (two amplified fragments of 324 and 223 bp corresponding to two different isoforms of VEGF); (*h*) Ang1, 5′-GAAGATATAACCGGATTCAAC-3′ and 5′-TGACAAGGTTATGAACTGTGT-3′ (amplified fragment of 698 bp); and (*i*) Ang2, 5′-ACTGACTGATGTGGAAGC-3′ and 5′-CTCTCAGTGCCTTGGAGTTAA-3′ (amplified fragment of 1131 bp).

Ten μl of each PCR product were electrophoresed in a 1.5% agarose gel in Tris/boric-acid/EDTA buffer, and then specific bands were analyzed using a program for densitometry kindly provided by P. L. Lollini (University of Bologna, Bologna, Italy).

Immunocytochemistry. Tumor cells grown on slides during cocultures were fixed in acetone for 10 min. They were then washed with PBS and incubated for 1 h at room temperature with anti-MIG (R&D Systems Inc., Minneapolis, MI); anti-GM-CSF (Genzyme, Milan, Italy); anti-iNOS (Tranduction Laboratories, Lexington, KY); anti-VEGF, anti-basic FGF, and anti-TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MIP-2 (Serotec Ltd., Oxford, United Kingdom); and anti-IP-10 (Peprotech Ec Ltd., London, United Kingdom). Hydrogen peroxide, normal goat blocking serum, biotinylated immunoglobulins, avidin-biotin complex, and fuchsin (Fuchsin Substrate Chromogen; Dako Spa, Milan, Italy) solutions were used according to the manufacturer's instructions (ABC ELITE detection system; Vector Laboratories, Burlingame, CA). Cells were lightly counterstained with Mayer's hematoxylin and mounted with Crystal/Mount (Biomeda, Foster City, CA). The positivity of the reactions was assessed independently in a blind fashion by two

[5] http://dscb041.sluigi.unito.it/DNA_MAP/DNA_MAP_help.htm.

pathologists on three samples from each experiment, and 100 consecutive cells were evaluated in three or more fields. The expression of cytokines was defined as absent (−) or scarcely (±), moderately (+), frequently (++), or strongly (+++) present on samples tested with the corresponding antibodies.

RESULTS

To evaluate the effect of IL-12 on the activation of T-LYs, Spcs from normal BALB/c and BALB/c-GKO mice were stimulated with anti-CD3 and anti-CD28 mAbs for 18 h in a complete culture medium supplemented or not supplemented with 10 ng/ml mouse recombinant IL-12. Whereas no significant variations in the cell surface differentiation and activation markers were found at the end of stimulation with or without IL-12, both normal and GKO LYs recovered from cultures with IL-12 displayed a 30% lower [^{3}H]thymidine uptake (Table 1). Moreover, normal IL-12-activated LYs produced 5-fold more IFN-γ, 20% less IL-2, and equal amounts of IL-4. LYs from GKO mice activated with or without IL-12 did not produce IFN-γ, whereas they released 3-fold more GM-CSF than normal LYs, as recently observed *in vivo* (19). The amounts of IL-10 and TNF-α produced by all these LYs were below the detection threshold (data not shown).

The ability of activated LYs cocultured in transwell inserts to modulate the gene expression of two distinct BALB/c mammary carcinoma cell lines [TSA (Ref. 11) and TUBO (Ref. 20)] growing at the bottom of wells was then evaluated. Cocultures with LYs activated in medium only were established in complete culture medium without IL-12, whereas those with LYs activated in the presence of IL-12 were established in medium supplemented with 5 ng/ml IL-12. After 96 h, modulation of gene expression was first assessed with the commercially available Atlas Mouse cDNA Expression Array from Clontech. With 2-fold expression as the cutoff threshold, TUBO cells cocultured with activated LYs from both normal and GKO mice up-modulated expression of the gene coding for the LPSR (CD14; LPSR, SwissProt accession number P10810; Ref. 21; Fig. 1, *TUBO CELLS*). By contrast, TUBO cells cocultured with normal IL-12-activated LYs up-modulated expression of LPSR and STAT1 (SwissProt accession number U06924), IRF-1 (SwissProt accession number M21065), and IP-1 (SwissProt accession number U19119). Expression of these three genes is regulated by IFN-γ secreted by IL-12-activated lymphoid cells (22). IFNγ absence when TUBO cells were cocultured with IL-12-activated GKO LYs further points to the central role of downstream IFN-γ in their up-modulation (Fig. 1, *TUBO CELLS*). By contrast, up-regulation of LPSR gene expression appears to be independent of both the downstream secretion of IFN-γ and the presence of IL-12. When the ability of activated LYs and IL-12-activated LYs to modulate gene expression in TSA cells was evaluated, LPSR gene up-modulation was no longer evident (Fig. 1, *TSA CELLS*). In the presence of IL-12-activated LYs, IP-1 and IRF-1 gene expression was

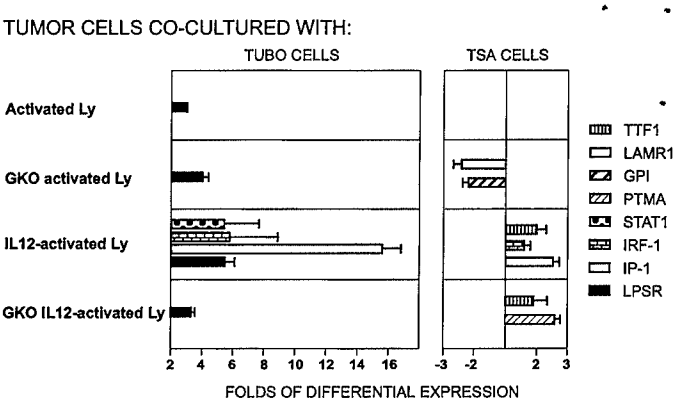

Fig. 1. Gene expression in TUBO cells and TSA cells recovered after 96 h of culture with activated and IL-12-activated LYs evaluated using the Atlas Mouse cDNA Expression Array.

up-regulated as seen in TUBO cells, although to a lesser extent. In this case as well, no up-regulated IRF-1 and IP-1 gene expression was found when TSA cells were cocultured with GKO IL-12-activated LYs (Fig. 1, *TSA CELLS*). The expression of transcription termination factor 1 (SwissProt accession number Q62187) was up-regulated when TSA cells were cocultured with both normal and GKO IL-12-activated LYs (Fig. 1, *TSA CELLS*). Interestingly, the absence of IL-12 and the absence of IFN-γ led activated LYs from GKO mice to down-regulate expression of the laminin receptor 1 gene (LAMR1, SwissProt accession number P14206), which is differently expressed during tumor invasion and metastasis (23), and the glucose-6-phosphate isomerase gene (GPI, SwissProt accession number P06745), which promotes the survival in culture of spinal neurons (24). No modulation of STAT1 gene expression was detected.

These data suggest that the factors released by activated and IL-12-activated LYs change the gene expression pattern in tumor cells. To overcome the rigidity imposed by the fixed cutoff chosen for evaluation of the macroarray data, the modulation of a few genes regulating key features of the tumor-host immune relationship was probed by semiquantitative RT-PCR, and the results were expressed as densitometric values (Table 2, A and B). A marked overexpression of the LMP2 and LMP7 genes and an increased expression of PA28 were found in TUBO cells cocultured with activated LYs from normal mice. The expression of these three immunoproteasome genes was further up-modulated when TUBO cells were cocultured with normal IL-12-activated LYs, whereas no up-modulation was found when they were cocultured with activated LYs and IL-12-activated LYs from GKO mice. A similar inhibition of LMP2, LMP7, and PA28 was observed when anti-IFN-γ mAb An18 was added at the beginning of the cocultures (data not shown). Whereas immunoproteasome genes

Table 1 *Features of LYs from normal and GKO mice recovered after 18 h of activation with anti-CD3 and anti-CD28 in the presence or absence of IL-12*[a]

	LYs from normal mice activated in		LYs from GKO mice activated in	
	Medium only	Medium with IL-12	Medium only	Medium with IL-12
[^{3}H]Thymidine uptake (cpm × 10^{-3})	114 ± 14[b]	88 ± 5[c]	103 ± 16	77 ± 5[c]
Cytokines in the supernatant				
IFN-γ (units/ml)	63 ± 34	297 ± 70[d]	<3	<3
IL-2 (pg/ml)	1395 ± 25	1144 ± 18[c]	1420 ± 9	1323 ± 40
GM-CSF (pg/ml)	50 ± 32	89 ± 25	252 ± 19[e]	380 ± 28[e]
IL-4 (pg/ml)	46 ± 3	38 ± 2	67 ± 4	58 ± 2

[a] No significant variations were found between activated LYs and IL-12-activated LYs for markers CD4, CD8, CD25, CD54, CD69, CD80, CD86, CD154, B220.2, STAT-1 Ph, STAT-3 Ph, H-2D^d, H-2K^d, and I-A^d.
[b] SD from the values in triplicate from three independent experiments.
[c] Significantly different ($P < 0.05$) from corresponding values displayed by activated LYs.
[d] Significantly different ($P < 0.001$) from corresponding values displayed by activated LYs.
[e] Significantly different ($P < 0.001$) from corresponding values for LYs from normal mice.

Table 2 *Modulation of gene and protein expression in TUBO and TSA cells cocultured with activated LYs and IL-12-activated LYs*

A. TUBO cells co-cultured with

			LYs from normal mice		LYs from GKO mice	
		Nothing	Activated Ly	IL-12-activated LYs	Activated LYs	IL-12-activated LYs
G3PDH	Gene[a]	1884	1889	1791	1949	1810
LMP2	Gene	1	70	237	5	13
LMP7	Gene	8	200	571	2	1
PA28	Gene	156	215	398	204	286
IP-10	Gene	20	441	576	148	95
	Protein[b]	±	++	+++	+	+
MIG	Gene	19	598	728	0	0
	Protein	±	++	+++	±	±
iNOS	Gene	3	161	223	44	115
	Protein	±	+	++	±	+
MCP1	Gene	28	18	58	17	25
Ang2	Gene	0	0	74	0	0
MIP-2	Protein	±	+	+	+	+
VEGF	Gene	53	37	13	34	71
	Protein	+	+	±	+	+
GM-CSF	Protein	−	−	−	−	−
TGF	Protein	±	+	+	+	+
FGF	Protein	±	+	+	+	+

B. TSA cells cocultured with

			LYs from normal mice		LYs from GKO mice	
		Nothing	Activated Ly	IL-12-activated LYs	Activated LYs	IL-12-activated LYs
G3PDH	Gene	1500	1501	1355	1471	1304
LMP2	Gene	35	37	90	28	53
LMP7	Gene	154	253	443	133	162
PA28	Gene	150	166	364	244	239
IP-10	Gene	5	245	669	268	267
	Protein	±	++	+++	++	++
MIG	Gene	0	2	802	0	0
	Protein	±	+	++	+	+
iNOS	Gene	0	161	249	130	133
	Protein	±	+	++	+	+
MCP1	Gene	21	116	256	119	34
Ang2	Gene	0	0	0	0	0
MIP-2	Protein	±	+	+	++	++
VEGF	Gene	162	76	41	103	168
	Protein	+	+	±	+	+
GM-CSF	Protein	−	−	−	±	±
TGF	Protein	+	+	+	+	+
FGF	Protein	+	++	++	++	++

[a] Evaluated by semiquantitative RT-PCR. Densitometric values were calculated as the intensity of the band obtained after the electrophoresis of the PCR products.
[b] Evaluated by immunocytochemistry. Stain intensity was defined as absent (−) scarce (±), moderate (+), frequent (++), and strong (+++).

are naturally expressed by TSA cells, their further up-regulation was found in the presence of normal IL-12-activated LYs but not in the presence of those from GKO mice. In both TUBO and TSA cells, the up-regulation of immunoproteasome genes increases with the increasing amount of IFN-γ in the culture medium and the membrane expression of MHC class I glycoproteins on tumor cells (data not shown). Expression of IP-10 and MIG, another two IFN-γ-inducible genes (25), is much more markedly up-modulated when TUBO (Table 2A) and TSA cells (Table 2B) are cocultured in the presence of IL-12-activated LYs than with activated LYs from normal mice. Surprisingly, the up-modulated expression of IP-10 in these tumor cell lines cocultured with activated LYs and IL-12-activated LYs from GKO mice was not inhibited, nor was it inhibited by the addition of An18 mAb (data not shown). These data suggest that factors other than IFN-γ released by activated lymphoid cells regulate IP-10 gene expression. A similar IFN-γ-independent regulation takes place with the iNOS gene. It was up-regulated after tumor cell coculture with activated LYs and further up-regulated by IL-12-activated LYs from normal mice. However, its up-regulation was also evident when TUBO cells were cocultured with activated LYs and IL-12-activated LYs from GKO mice (Table 2A). Expression of MCP1 is markedly up-modulated in TUBO and TSA cells cocultured with normal IL-12-activated LYs and not in those cocultured with GKO IL-12-activated LYs. It is also up-modulated in TSA cells cocultured with activated LYs (Table 2B). Expression of VEGF, a factor of crucial importance for tumor angiogenesis (26), is markedly down-modulated in tumor cell lines cocultured with IL-12-activated LYs and, to a lesser extent, with activated LYs. Lastly, in TUBO cells cocultured with IL-12-activated LYs only, expression of Ang2 (26, 27) became evident. No down-modulation of VEGF gene expression or up-modulation of Ang2 gene expression was found when tumor cells were cocultured with activated LYs and IL-12-activated LYs from GKO mice (Table 2, A and B). In both cases, Ang1 expression remained undetectable (data not shown).

In a blind fashion, two trained pathologists also evaluated whether the up-modulated expression of a few genes correlated with the overexpression of the proteins they encode, as assessed by immunocytochemistry. On culturing TUBO and TSA cells on microscope slides placed at the bottom of the transwells in cocultures with activated LYs and IL-12-activated LYs, it was found that the up-modulation of MIG and IP-10 gene expression detected by semiquantitative RT-PCR fit in well with the intensity of protein expression (Fig. 2; Table 2, A and B). Immunocytochemistry data also endorse the indication of an IFN-γ-independent up-regulation of IP-10 but not

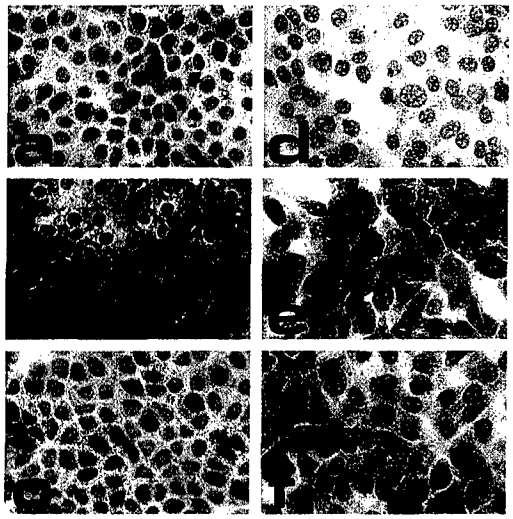

Fig. 2. Immunocytochemistry evaluation of MIG and IP-10 expression in TUBO (*a–c*) and TSA (*d–f*) cells recovered from 96-h cultures. MIG expression is scanty in TUBO cells cultured alone (*a*) or cocultured with IL-12-activated LYs from GKO mice (*c*), whereas it is strong in TUBO cells cocultured with IL-12-activated LYs from normal mice (*b*). IP-10 expression is also weak in TSA cells cultured alone (*d*) but is marked in TSA cells cocultured with IL-12-activated LYs from normal (*e*) and GKO (*f*) mice (original magnification, ×1000).

of MIG expression when tumor cells are cocultured with activated LYs and IL-12-activated LYs from GKO mice. A similar activated and IL-12-activated LY-dependent but IFN-γ-independent up-regulation is also suggested for MIP-2, TGF, and FGF protein expression. In the absence of IFN-γ, MIP-2 is much more strongly expressed by TSA cells (Table 2B). Lastly, VEGF is poorly expressed in tumor cells cultured with IL-12-activated LYs.

DISCUSSION

The data reported here show that these two distinct lines of mammary carcinoma cells change gene and protein expression when cocultured in the presence of activated T-LYs. This finding suggests a new way in which the immune system affects the growth of a tumor so that it becomes a party to its own inhibition.

The amount of IFN-γ released by T-LYs appears to be of major importance in the gene and protein modulation in tumor cells. This finding fits in well with the strong influence that IFN-γ has on the expression of a great number of genes. Indeed, the greater ability of IL-12-activated LYs to modulate the genetic program of tumor cells appears to rest on IL-12's ability to induce the prolonged release of large amounts of IFN-γ in normal T-LYs. However, the present data lead to the rather obvious conclusion that induction of a high and prolonged IFN-γ release results in marked gene modulation in tumor cells and disclose a rather complex pattern of gene modulation by T-LYs. Parallel cocultures of tumor cells with activated and IL-12-activated LYs from normal and GKO mice revealed distinct categories of genes. The first group embraces genes (STAT1, IRF-1, LMP2, LMP7, MIG, MCP1, and Ang2) that are mostly up-modulated by high amounts of IL-12-induced IFN-γ. These genes are up-modulated in tumor cells from cocultures with normal activated LYs, more so in cells from cocultures with normal IL-12-activated LYs and not at all in cocultures with both activated and IL-12-activated LYs from GKO mice and with normal IL-12-activated LYs in the presence of anti-IFN-γ An18 mAb. Other genes (PA28, IP-10, iNOS, and MIP-2) are overexpressed in the presence of IFN-γ even if their expression is still enhanced in cocultures with activated and IL-12-activated LYs from GKO mice and in those in the presence of anti-IFN-γ An18 mAb. This suggests that IFN-γ is not the only factor released by activated LYs that is implicated in the up-regulation of their expression (19). IP-10 can also be modulated by IFN-α and IFN-β (28), which are not neutralized by An18 mAb. The third group embraces genes (LPSR, transcription termination factor 1, TGF, and FGF) whose expression is promoted by factors released by activated LYs apart from the presence or absence of IFN-γ. In contrast, in the absence of factors released by normal activated LYs, the expression of the LAMR1 and GPI genes is inhibited. Distinct factors, such as those released by GKO IL-12-activated LYs, may restore their expression to normal levels.

Because immunocytochemistry detects only relatively high protein levels, it is conceivable that any modulation in protein expression detected may have a functional consequence. The modulation of a few genes and/or the overexpression of their protein product take place in both TUBO and TSA carcinoma cells (IP-10, MIG, iNOS, MCP1, MIP-2, VEGF, and FGF). LPSR and STAT1 genes are modulated in TUBO cells, but not in TSA cells. Modulation of LAMR1 and GP1 occurs the other way around, pointing to the idiosyncratic features of each tumor cell line. The activation of LMP1, LMP7, and PA28 in TUBO cells cocultured with IL-12-activated LYs implies their critical passage from normal to immunoproteasome (29). This passage is not evident in TSA cells because they constitutively express the immunoproteasome.

The gene modulations described seem to be a consequence of the factors released by activated T-LYs. The repertoire, the amount, and the persistence with which these factors are released are influenced by the presence or absence of cytokines during LY activation. The sole presence of IL-12 never affected gene and protein expression in TUBO and TSA cells, nor was gene modulation found in tumor cells recovered after 4 and 96 h of culture in medium only (data not shown). In contrast, it markedly increased the release of IFN-γ in normal activated LYs and that of GM-CSF in GKO activated LYs. The presence of the whole Spc population during T-LY activation allows these IL-12-induced cytokines to promote the release of several additional downstream factors that are not necessarily directly released by T-LYs. In effect, the reaction activated *in vivo* by IL-12 involves many types of cells and factors, in both the presence (11, 14) and the absence (19) of secondary IFN-γ.

Among the events activated by IL-12, the modulation of gene expression by tumor cells may play a significant role (14). Whereas the purpose of this study was to point out this new way in which lymphoid cells may interfere with tumor growth, the pattern of down-modulation of VEGF observed in TUBO and TSA cells and the up-modulation of Ang2, MIG, IP-10 and iNOS fit in well with the inhibition of neoangiogenesis and the damage of neoformed vessels that characterize the antitumor reaction activated by IL-12 leading to ischemic necrotic tumor rejection (11, 12). High Ang2 expression in tumor vessels causes their destabilization and regression, perhaps as part of the host reaction (27). On the other hand, tumor-derived VEGF represses these Ang2 regression signals (30). In our cocultures, induction of Ang2 expression coincided with VEGF down-modulation, thus resulting in a strong antiangiogenic setting. Up-modulation of MIG1, IP-10, and iNOS stresses this antiangiogenic scenario even further.

It is not surprising that the number of genes modulated by IL-12-activated LYs is low because it has been recently shown that independent samples taken from the same tumor after surgery, chemotherapy, and metastasis retain the same gene expression pattern (31). Whereas the consequences of other gene modulations in shaping tumor growth and inhibition and tumor interaction with immune cells are still speculative, a few changes in the tumor genetic program induced by IL-12-activated LYs may be truly important because they

enroll tumor cells themselves in IL-12-activated tumor inhibition. IL-12-induced reaction very effectively inhibits TSA cell growth (11) as well as that of many other tumors (10). It is less effective on TUBO cells (data not shown), whereas it marginally affects the growth of a few other tumors (10, 11). These outcomes may be influenced by differences in the pattern of tumor gene modulation that may well be induced by IL-12-activated LYs on individual tumors.

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Advances in Brief

A Light, Nontoxic Interleukin 12 Protocol Inhibits HER-2/*neu* Mammary Carcinogenesis in BALB/c Transgenic Mice with Established Hyperplasia[1]

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Abstract

With a slight asynchronous but consistent progression, all of the mammary glands of female BALB/c mice transgenic for the transforming rat HER-2/*neu* oncogene progress to atypical hyperplasia and to invasive carcinoma. Previous studies have shown that chronic administration of interleukin (IL) 12 started at the 2nd week of age hampers this progression because of its ability to inhibit tumor angiogenesis and activate a nonspecific immune response. Here we show that a similar inhibition is achieved when 7-week-old mice with fully blown atypical hyperplasia receive a weekly injection of 100 ng IL-12 for 16 times. This lower-dose and later IL-12 administration induces high and sustained levels of serum IFN-γ equivalent to those elicited by more frequent administrations. A lower-dose and less toxic treatment may thus be envisaged as a possible option in the management of preneoplastic mammary lesions.

Introduction

The effectiveness of systemic (1) and local (2) IL-12[3] in inhibiting mammary tumors seems to rest on its ability to trigger the inhibition of tumor angiogenesis by secondary cytokines and third-level chemokines, to activate CTL and leukocyte subsets capable of producing proinflammatory cytokines, and finally to induce polymorphonuclear cells to destroy neoformed tumor vessels (3, 4). The combination of these activities leads to the rejection of transplantable tumors (1, 4) and the inhibition of chemically induced carcinogenesis (5) and HER-2/*neu* oncogene-dependent mammary carcinogenesis in transgenic mice (6). Inhibition of HER-2/*neu* carcinogenesis is particularly effective when IL-12 treatment is begun during the very early stages of carcinogenesis and tapers off when it is started at week 13 (7). In addition to timing, the IL-12 dose is another critical issue. Inhibition was achieved with 5-day injections of 100 ng IL-12, whereas doses 10 and 50 times lower were almost ineffective (7).

Extrapolation of these previous findings to a clinical setting suggested that IL-12 treatment may 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 per body weight and the heavy schedule of administration would hinder the use of IL-12 in the prevention of human mammary tumors (8).

We show here that a much lower dose of IL-12 that is started when adult mice already present full-blown atypical mammary hyperplasia is as efficacious as much earlier and heavier treatments.

Materials and Methods

Mice. Inbred female BALB/c mice overexpressing the transforming rat HER-2/*neu* oncogene (*neu*T$^+$/*neu*T$^-$) driven by the mouse mammary tumor virus promoter (BALB-neuT) and transgene negative (*neu*T$^-$/*neu*T$^-$; BALB/c) were bred under specific pathogen-free conditions by Charles River (Calco, Italy), screened for the presence of the transgene as previously described in detail (8), and treated in accordance with European Union and institutional guidelines. Because all of the 10 mammary glands of BALB-neuT females undergo carcinogenic transformation with a definite progression (6, 9), these were inspected weekly, and tumor masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all of the mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, at which time mice were killed for humane reasons. Surviving mice were killed at 33 weeks (6). Because some immunized mice do not display carcinomas in all of the mammary glands, the mean number of palpable mammary carcinomas per mouse was calculated as the cumulative number of incidents tumors/total number of mice.

IL-12 Administration. Recombinant IL-12 (Genetics Institute, Cambridge, MA) in PBS supplemented with 0.01% MSA (Sigma, St. Louis, MO) was administered i.p. Starting from the 7th week of age BALB-neuT mice received weekly i.p. injections of 0.2 ml of PBS containing MSA only (MSA controls) or MSA plus 100 ng of IL-12, for a period of 4 weeks, followed by a 3-week rest. This course was repeated either once, twice, or three times (Fig. 1). Another group of mice remained untreated. Because no appreciable differences in tumor growth rate and pathological findings were found between the untreated mice and the MSA controls, only the data of the latter group are shown. In another set of experiments, BALB/c mice treated i.p. with IL-12 were killed at various times after treatment to test the IFN-γ titer in sera and the ability of Spc to produce IFN-γ after polyclonal activation. These mice were injected daily for 1–5 days or weekly for 1–3 weeks.

Histological and Immunohistochemical Analysis. Groups of three IL-12-treated and MSA-treated BALB-neuT mice were killed at progressive times. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E or Giemsa and the trichrome method.

Preparation and Activation of Spc. Seven days after the beginning of treatment, Spc from BALB/c mice untreated or injected one to five times with 100 ng of IL-12 were suspended at 2×10^6/ml in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) with 50 μg/ml gentamicin (BioWhittaker Europe) and 10% heat-inactivated FCS (RPMI complete medium; Life Technologies) with or without 2 μg/ml ConA and used for the ELISPOT assay.

ELISPOT Assay. Ninety-six-well MultiScreen Filtration plates (Millipore S.A., Molsheim, France) were coated with capture anti-IFN-γ monoclonal antibody (R46A2; Endogen, Woburn, MA) at 5 μg/ml in PBS overnight at

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[3] The abbreviations used are: IL, interleukin; MSA, mouse serum albumin; Spc, total spleen cells; ConA, concanavalin A.

Appendix 11

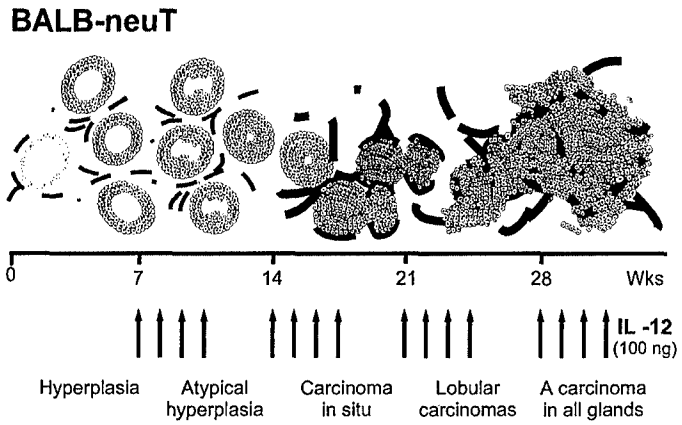

Fig. 1. Outline of the times of the 16 IL-12 injections during the progression of the natural carcinogenesis in all of the 10 mammary glands of BALB-neuT mice. Each *arrow* represents one weekly i.p. injection of 100 ng IL-12. With a slightly asynchronous but consistent pattern, all of the mammary glands of BALB-neuT female mice progress to invasive carcinoma, starting from atypical hyperplasia by week 2. This atypical hyperplasia becomes prominent and displays vigorous capillary proliferation by week 14 and then assumes the appearance of a carcinoma *in situ* that progressively expands and by week 21 invades the surrounding tissues. At the 33rd week, all of the 10 glands have palpable tumor masses.

4°C. They were then blocked with PBS 5% BSA and washed with PBS. Two hundred thousand Spc or ConA-activated Spc were added to each well in 100 μl of complete RPMI. After 48 h of incubation at 37°C and 5% CO_2, the plates were washed with PBS 0.05% Tween 20 (Sigma), and biotinylated detection anti-IFN-γ monoclonal antibody (XMG1.2; Endogen) at 1 μg/ml in PBS 2% BSA was added and incubated overnight at 4°C. One hundred μl of a 1:6000 dilution of streptavidin-horseradish peroxidase (N-200; Endogen) in the appropriate buffer (N-500, Endogen) were then added and incubated for 1 h at room temperature. The spots were developed with the AEC101 kit (Sigma) according to the manufacturer's instructions and counted on a computer-assisted ELISPOT image analyzer AID (AID ELISPOT Version 2.0; Autoimmun Diagnostika GmbH, Strassberg, Germany).

IFN-γ Titration in Sera. Serum samples from BALB/c mice injected daily for 1–5 days or weekly for 1–3 weeks with 100 ng of IL-12 were collected at the indicated time points and assayed for the presence of IFN-γ with a sandwich ELISA kit (PharMingen, San Diego, CA).

Statistical Analysis. Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test; differences in the number of tumors per mouse and the number of spots in the ELISPOT test were evaluated by Student's *t* test.

Results

Delayed Carcinogenesis in Mice Receiving IL-12. The aggressive mammary carcinogenesis that takes place in all of the mammary glands of BALB-neuT female mice (Fig. 1) was significantly hampered in those receiving 16 i.p. administrations of 100 ng IL-12 divided into four courses of weekly injections for 4 weeks, followed by a 3-week rest (Figs. 1 and 2, *A* and *B*). Both a delay in the onset of the first mammary tumor (Fig. 2*A*) and a reduction in the number of mammary glands with a palpable tumor at 33 weeks were found (Fig. 2*B*). All of the IL-12-treated mice were free of palpable tumors at 20 weeks, when >50% of control mice already displayed palpable tumors. At week 24, all of the control mice displayed tumors, whereas 76% of the treated mice were still completely tumor free (Fig. 2*A*). The number of tumors per mouse was also significantly lower in the IL-12-treated mice (Fig. 2*B*). Statistical analysis showed that the whole progression of carcinogenesis was significantly delayed ($P < 0.0001$). To asses if all of the four courses of IL-12 treatment were necessary for the effective tumor inhibition, in another set of experiments BALB-neuT mice received only the first two or three courses of IL-12. Although three courses were still effective, though to a lesser extent, two courses delayed the onset of the first mammary tumor (Fig. 2*A*), but all of the glands had a palpable tumor at 33 weeks (Fig. 2*B*).

Pathological Findings. A histological examination of mammary tissue of 7-week-old mice showed widespread atypical hyperplasia of small lobular ducts and lobules characterized by a proliferation of a relatively uniform population of round epithelial cells that assumed a stratified appearance and a solid, occlusive growth. Numerous enlarged capillaries were present in the loose stroma surrounding the hyperplastic foci (Fig. 3*a*). At week 33, when all of the 10 glands of MSA-treated mice displayed large, invasive lobular carcinomas (Fig. 3*b*), ~50% of those of the IL-12-treated animals showed only multiple foci of atypical hyperplasia surrounded by a dense and fibrotic stroma with an evident reactive cell infiltrate (Fig. 3, *c* and *e*). A dense stroma was also interposed among the neoplastic alveolar nodules of the invasive lobular carcinomas observed in the other 50% (Fig. 3, *d* and *f*). The neoplastic epithelial cells of these carcinomas were often necrobiotic. Tumor growth thus lacked cohesion and was marked by the presence of fissures filled with necrotic and hemorrhagic material.

IFN-γ Production by IL-12-treated Mice. These data indicate that inhibition of carcinogenesis after weekly IL-12 injections is no less marked than previously observed with five injections per week (7). Because the ability of IL-12 to elicit high levels of IFN-γ correlates with the clinical response (9), serum IFN-γ levels after a single and multiple IL-12 injections were compared. To avoid possible immunosuppressive activities related to the progression of mammary carcinogenesis, neuT$^-$/neuT$^-$ BALB/c mice were used. Within 6 to 120 h after a single IL-12 injection, the IFN-γ titers were much higher than for the same period after the fifth IL-12 injection (Fig. 4, *A* and *C*). Moreover, although 6 h after one or two IL-12 injections high titers of IFN-γ were found, these dropped progressively by further increasing the number of IL-12 injections (Fig. 4*B*). However,

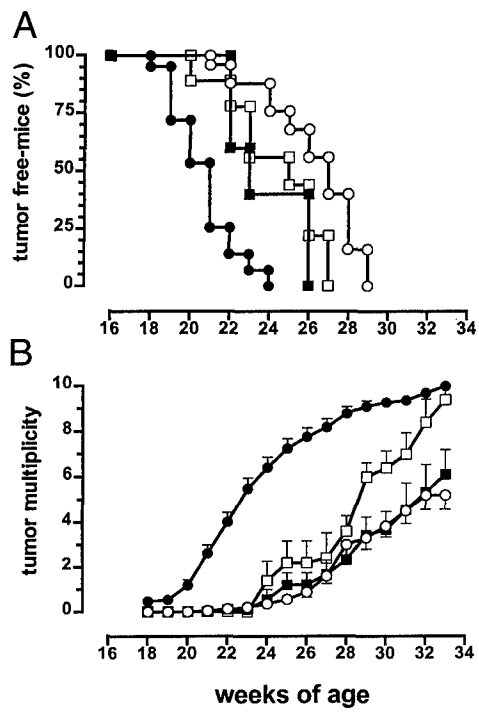

Fig. 2. Progression of mammary carcinogenesis in BALB-neuT mice receiving IL-12 injections. *A*, percentage of tumor-free mice; *B*, tumor multiplicity, calculated as the cumulative number of incident tumor/total number of mice. There were 43 mice in the MSA control group (●) and 25 mice in the group that received the complete IL-12 treatment (○; see "Materials and Methods"). Nine and five mice were in the groups that received only the first two (□) and three (■) courses, respectively, of the IL-12 treatment.

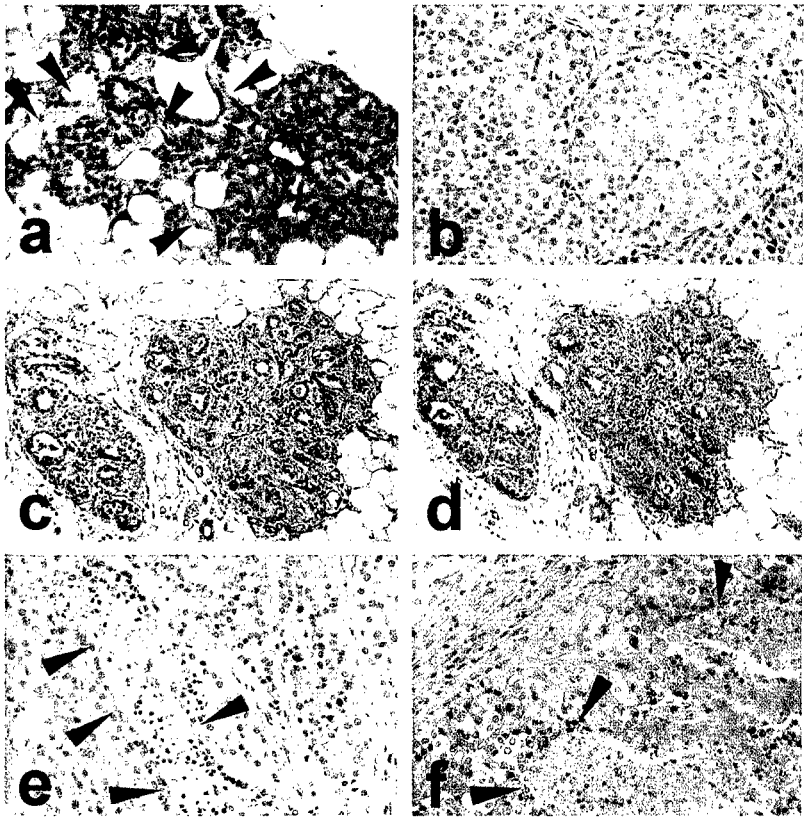

Fig. 3. Histology of mammary tissue obtained from untreated (*a* and *b*) and IL-12 -treated (*c–f*) BALB-neuT mice. At 7 weeks of age, untreated mice show several foci of mammary ductal-lobular hyperplasia characterized by a pluristratified epithelium sometimes occluding the ductal-lobular structures (*a*). Numerous enlarged capillaries are present in the delicate stroma (*arrowheads*). At 33 weeks of age, when all of the 10 glands of untreated mice display large invasive lobular carcinoma (*b*), ~50% of those of the IL-12-treated animals show multiple foci of atypical hyperplasia surrounded by a dense fibrotic stroma (*c*), easily recognizable by the *blue-green* staining evidenced by the trichrome method (*d*). A dense stroma is also interposed among the neoplastic epithelial aggregates of the invasive lobular carcinoma observed in the other 50% (*E*, H&E; *f*, trichrome method). The numerous necrotic foci (*arrowheads*) disaggregating the tumor as well as the intracytoplasmic vacuolizations present in the neoplastic epithelial cell are signs of severe ischemic damage. (*a*, *b*, *e*, and *f*, ×400; *c* and *d*, ×200).

when mice that received one IL-12 injection were boosted 7 and 14 days later, the levels of IFN-γ decreased (Fig. 4*D*).

The IFN-γ production was evaluated in an ELISPOT assay by a collection of Spc 7 days after the last IL-12 injection and stimulating them with ConA (Fig. 5). A large number of spots were displayed in

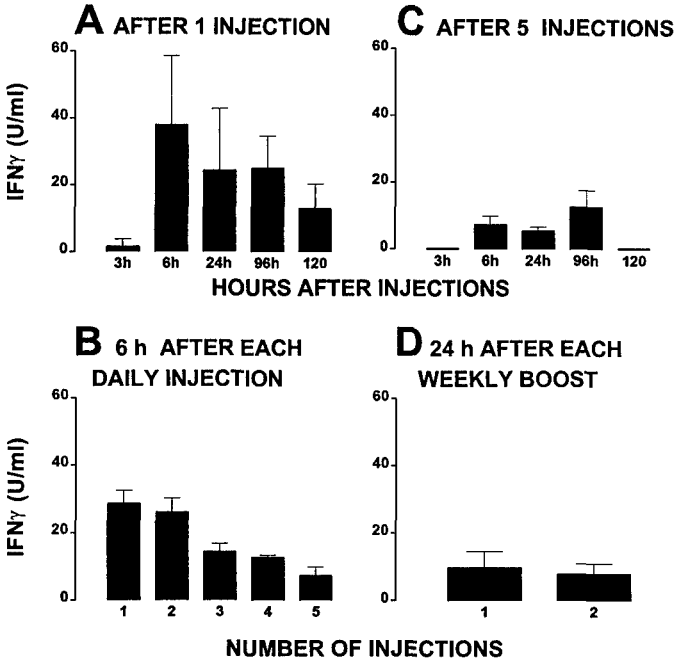

Fig. 4. IFN-γ titers in sera from BALB/c mice injected i.p. with 100 ng of IL-12. Titers in sera from mice: injected once and collected at progressive intervals (*A*); injected daily for 1–5 days and collected 6 h after each injection (*B*); injected daily for 5 days and collected at progressive intervals after last injection (*C*); injected once, boosted 7 and 14 day later, and collected 24 h after each booster (*D*).

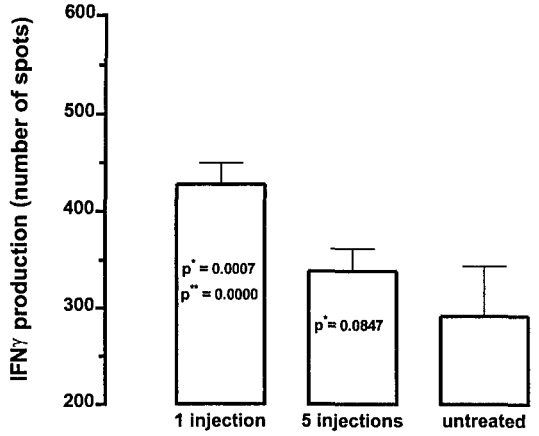

Fig. 5. IFN-γ production by stimulated Spc from untreated mice or mice injected one or five times with IL-12, assessed by ELISPOT assay. Spc were stimulated as described in "Materials and Methods." Results are expressed as the mean number of spots ± SD of samples from at least three mice per treatment group independently tested. *, *P*: significance *versus* untreated mice; **, *P*: significance *versus* mice injected five times.

Spc from mice injected only once, whereas that displayed by Spc from mice injected five times differed only slightly from that of Spc from control untreated mice (Fig. 5).

Discussion

Present data show that 16 weekly injections of IL-12 significantly hamper the progression of the very aggressive mammary carcinogenesis driven by the activated rat HER-2/*neu* oncogene, which results in the establishment of a large, fast-growing, metastasizing lobular carcinoma in all of the 10 mammary glands of BALB-neuT mice by the 33rd week of age (6, 10). These mice, in fact, are not simply at risk but are genetically predestined to develop multiple tumors (11).

The path of this carcinogenesis leads through an initial oncogene product expression leading to hyperplasia. The second stage consists in the induction of angiogenesis. A close connection between atypical hyperplasia and the activation of angiogenesis is evident: at 7 weeks, widespread atypical hyperplasia accompanies flourishing neovascularization (10). The success of IL-12, indeed, may well be partly attributable to its commencement at the time of this close connection and hence before overt tumor formation (7). At 33 weeks, ~50% of the glands of IL-12-treated mice were tumor free and showed a fibrosis around the persisting but impoverished hyperplastic foci. This fibrosis was the consequence of the chronic and persistent inflammatory, antiangiogenic reaction induced by IL-12. The necrobiotic appearance of the neoplastic epithelial cells and the numerous foci of ischemic necrosis found in the invasive lobular carcinomas of IL-12-treated mice are equally attributable to the several activities induced by IL-12. These and previous data on HER-2/*neu* transgenic mice (6), along with those concerning 3-methylcholanthrene carcinogenesis (5), emphasize the significance in tumor prevention (8) of the concurrence of nonspecific immunity and antiangiogenesis elicited by IL-12.

The HER-2/*neu* oncogene is overexpressed in a substantial proportion of human mammary carcinomas (12). The close resemblance of the progression of mammary carcinogenesis in HER-2/*neu* transgenic mice to that in women (10) suggests that administration of IL-12 may be a significant prophylactic strategy. Early IL-12 administration would seem unnecessary, because the present findings show that it is still very effective if commenced when widespread atypical hyperplasia is already evident in all of the 10 mammary glands. In a human setting, it might thus be possible to start IL-12 administration when an overt preneoplastic lesion is evident and not to confine it to healthy persons with a genetic risk (13). 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 (6, 7) with no loss of efficacy. The present IL-12 administration schedule seems to escape the dose-dependent transient suppression of the immune response (14, 15) that accompanies chronic administration of more frequent injections (7). It also induces equivalent high, sustained serum IFN-γ levels. This is an important issue, because the ability to maintain IFN-γ induction appears to be associated with the clinical response (9).

The use of IL-12 in humans is complicated by schedule- and dose-dependent toxicity (9, 16). Weekly administration of 500 ng/kg is well tolerated by melanoma patients. It is effective clinically (9) and transiently boosts tumor-specific T lymphocytes (17). Unfortunately, even with the present light schedule, the single dose effective in mice is still 8–10 times higher than the 500–700 ng/kg tolerated by humans, and lower single doses are almost ineffective (7). For other cytokines (*e.g.*, IL-2), increased experience and refinements in patient selection and administration schedules have greatly increased the safety of antitumor regimens (18). In any event, cancer prevention is another matter, and comparison with the doses used in cancer therapy may be inappropriate. For example, in the case of estrogen receptor modulators such as tamoxifen or raloxifene, the doses used in tumor-free individuals for prevention or for adjuvant therapy of breast carcinoma are about 10 times lower than those used to treat advanced or metastatic tumors (19, 20).

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RESEARCH ARTICLE

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

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An assessment was made of the effectiveness of DNA vaccination in prevention of the mammary adenocarcinomas of BALB/c female mice transgenic for the activated rat Her-2/neu oncogene. Atypical hyperplasia is evident in their mammary glands when they are 6 weeks old and in situ carcinoma by the 13th week. Palpable invasive carcinomas appear around the 17th week and are always evident in all 10 glands by the 33rd week. Intramuscular vaccinations with 100 μg plasmid DNA encoding the extracellular domain of the Her-2/neu p185 (ECD) performed at the 6th, 12th, 18th and 24th week provided no significant protection, whereas those ECD plasmids in which the DNA coding for the immunomodulatory 163–171 (VQGEESNDK) nonapeptide of human IL1β (ECD-IL1βp) had been inserted both delayed carcinogenesis and reduced tumor multiplicity. This reduction was associated with a marked immune-inflammatory reaction and a conspicuous leukocyte infiltrate located in the stroma surrounding the hyperplastic mammary ductul-alveolar structures. It was also directly correlated with a high anti-p185^{neu} antibody production and an immunoglobulin switch to IgG2a and IgA. No anti-p185^{neu} cytotoxic response was found. No significant protection was obtained when the DNA coding for the non-active peptide 189–197 of IL1β was inserted. Gene Therapy (2001) **8,** 447–452.

Keywords: *IL-1β; DNA immunization; mammary carcinogenesis; Her-2/neu; tumor prevention*

Introduction

DNA vaccines are molecularly defined reagents that are easy to construct and elicit long-lasting cellular and humoral immune responses to a variety of antigens. Clinical trials have shown that they are nontoxic and well tolerated, though the responses their vaccination induce are low and vary from one individual to another,[1–3] while their efficacy is often limited by low levels of gene and protein expression and the complex requirements for protein presentation and lymphocyte activation.[4]

Enhancement of the potency of DNA vaccines has been sought through the employment of costimulatory molecules and cytokines as adjuvants.[5,6] Vaccines encoding antigens fused with immunological molecules and cytokines elicit more effective responses[6,7] and the ability of cytokines to enhance the immune recognition of tumor antigens has been extensively exploited.[7] Cytokine-gene engineered tumor cells,[8] and DNA encoding fusion proteins between cytokines and tumor antigens[9] induce marked immune responses, even against poorly immunogenic tumors.

Interleukin 1 (IL-1) is a particularly effective adjuvant,[10] but its potent pyrogenic and/or proinflammatory properties drastically limit its use. The nonapeptide sequence VQGEESNDK corresponding to the amino acid stretch between the positions 163–171 of human IL-1β, on the other hand, is free from these properties and retains the immunostimulatory capability of the entire molecule.[11,12] Its local administration, in fact, markedly increase the immunogenicity of poorly immunogenic tumors in syngeneic mice.[12] Insertion of the DNA sequence encoding this nonapeptide in recombinant antigens enhances their immunogencity.[13] DNA vaccination with plasmids encoding a fusion protein between idiotypic determinants of B cell lymphomas and this peptide induced a protective immune response against a subsequent lymphoma challenge.[14]

In this paper, we compare the ability of DNA vaccination with plasmids coding for the extracellular domain of product of rat Her-2/neu (p185^{neu}) alone (ECD) or fused with the DNA coding for this IL-1β peptide (ECD-IL1βp) to block the progression of Her-2/neu carcinogenesis in female BALB/c mice transgenic for the activated rat Her-2/neu oncogene under the control of the MMTV promoter (BALB-neuT).[15] All the mammary glands of these mice independently undergo a very aggressive carcinogenesis that mirrors some features of the formation of lobular carcinoma in women.[16] Vaccination with plasmids coding for ECD alone did not block this carcinogenesis, whereas vaccination with ECD-IL-1βp was followed by a significant delay.

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Results

Immunization of BALB-neuT mice

Owing to expression of the activated rat Her-2/neu gene under the control of the MMTV promoter, high membrane overexpression of rat $p185^{neu}$ is already evident in the terminal ductular–lobular units of all the mammary glands of BALB-neuT female at the 3rd week of age. Atypical mammary hyperplasia is evident between week 3 and 6. Palpable invasive carcinomas appear around the 17th week in one or two glands of 40% of mice and are always evident in all 10 glands by the 33rd week.[15,16]

To assess the ability of DNA vaccination to hamper this progression, mice were immunized at the 6th, 12th, 18th and 24th week with plasmids coding for the ECD alone, ECD and IL-1βp (ECD-ILβp), or ECD and the non-active peptide 189–197 of IL-1β (ECD-IL1βna). Vaccination with these plasmids did not hamper tumor growth, whereas immunization with ECD-IL1βp both delayed carcinogenesis and reduced tumor multiplicity (Figure 1). The mean number of mammary glands with palpable tumors at this time was six only in the ECD-IL1βp group, whereas all mice in the other groups displayed a palpable tumor in all their mammary glands (Figure 1, lower panel). Moreover, one of the 10 mice in the ECD-IL1βp group was still completely tumor free at week 33 (Figure 1, upper panel).

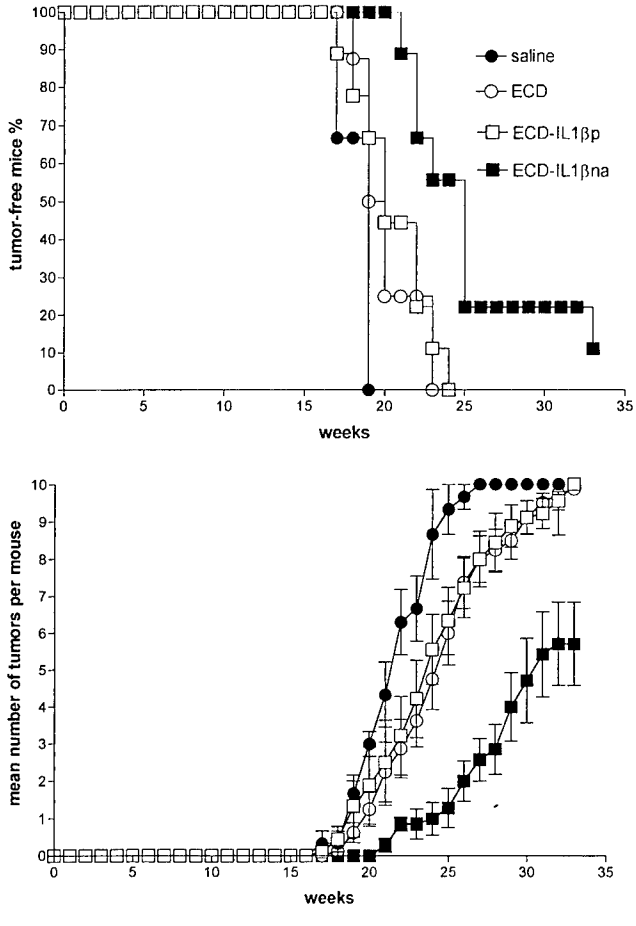

Figure 1 *Effect of vaccination with plasmids coding for ECD alone or fused with IL-1βp on the mammary carcinogenesis in BALB-neuT mice. The time of appearance of the first tumor (upper panel) and mean number of palpable mammary carcinomas per mouse (lower panel) in the group of eight mice injected with saline only (●), or vaccinated with ECD (○), ECD-IL1βna (□), and in a group of 10 mice immunized with ECD-IL1βp (■).*

Pathological analysis of mammary glands

Pathological observations performed at 7 weeks, 1 week after the first immunization showed that both mice injected with saline and immunized with ECD-IL1βp displayed foci of epithelial hyperplasia of the terminal ductular-lobular units (TDLU) (Figure 2). However, in immunized mice the TDLU were surrounded by a reactive leukocyte infiltrate (Figure 2b). At 13 weeks, 1 week after the second immunization mice injected with saline displayed a well-developed atypical epithelial hyperplasia. Epithelial cells were atypical and their growth inside the lumens distended and expanded the alveoli and lobules (Figure 2c). By contrast, mice immunized twice with ECD-ILβp showed reduced hyperplasia and infiltrating reactive cells in the surrounding fibrotic stroma (Figure 2d). At 25 weeks, 1 week after the third immunization, a well-developed invasive lobular carcinoma was present in most mammary glands from control mice (Figure 2e), while only several foci of atypical hyperplasia and *in situ* carcinoma were found in some mammary glands of immunized mice (Figure 2f). These

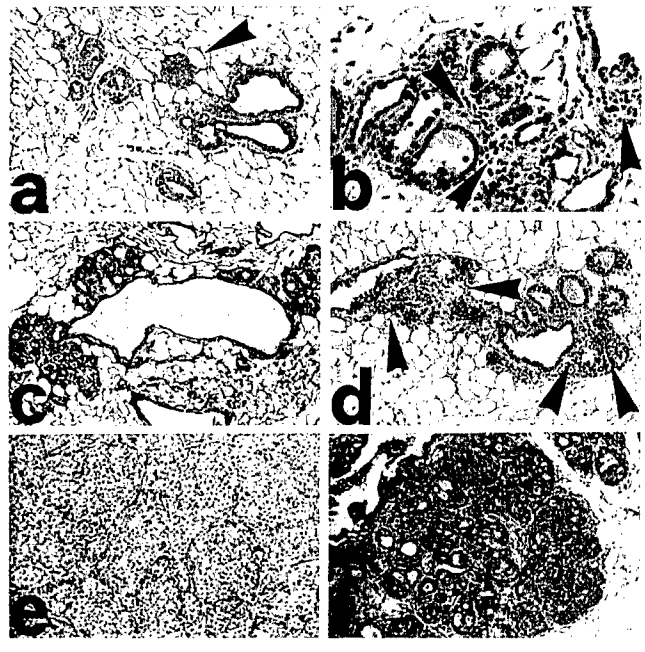

Figure 2 *Histology of mammary tissue obtained from mice injected with saline (a, c, e) or immunized with ECD-IL1βp (b, d, f). At 7 weeks of age, mice injected with saline (a) show several foci of mammary ductul-lobular hyperplasia constituted by a pluristratified epithelium sometimes occluding the alveolar lumen (arrowhead). By contrast the mammary tissue of immunized animals (b) shows that the ductul-alveolar structures, mainly lined by a single epithelial cell layer, are clearly surrounded by lymphoid reactive cells (arrowheads). Thirteen-week-old mice injected with saline develop multifocal atypical hyperplasia (c) with several alveolar structures often filled by transformed epithelial cells. At the same age in immunized mice the hyperplasia is less pronounced and accompanied by an evident reactive cell infiltrate localized in the surrounding fibrotic stroma (arrowheads) (d). At 25 weeks of age, a well-developed invasive lobular carcinoma was present in several mammary glands of mice injected with saline (e) while, in those from immunized mice (f) hyperplasia with foci of in situ carcinoma bordered by reactive cell infiltrated fibrotic stroma was the prevalent pathological feature (a, c–f ×200; b ×400).*

foci were bordered by a dense stroma markedly infiltrated by reactive leukocytes.

Cytotoxic response to p185^{neu} positive target cells

The infiltrate and inhibition of the progression of carcinogenesis did not correlate with the induction of a detectable cytotoxic response in Spc collected 7 days after each vaccination and tested immediately or after 6 days *in vitro* restimulation with distinct p185^{neu+} target cells, as evaluated in 4 and 18 h ^{51}Cr release assays and 48 and 72 h [^{3}H]TdR release assays (data not shown).

Antibody response associated with the inhibition of natural carcinogenesis

The ability of these treatments to induce anti-p185^{neu} antibodies was evaluated in the sera collected at 33 weeks when all the mice vaccinated with saline, ECD or ECD-IL1βna displayed 10 large tumors. The antibody titer in mice with 10 large tumors was similar, irrespective of the vaccination with ECD, ECD-IL1βna or ECD-IL1βp. By contrast, the titer was much higher in the sera from mice vaccinated with ECD-IL1βp with only 1–4 tumors (Figure 3).

The distribution of immunoglobulin isotypes was also evaluated in these sera. IgM and IgG3 were increased in all immunized mice (Figure 4). In addition, mice with 1–4 tumors after ECD-IL1βp immunization presented an increase of several isotypes, especially IgG2a and IgA.

Discussion

Present data show that insertion of IL-1βp DNA dramatically increases the protective efficacy of vaccination with plasmids coding for ECD. Only mice immunized with ECD-IL1βp plasmid display a delay in the appearance of the first tumor and a strong decrease of the number of mammary glands with a palpable carcinoma, whereas the natural consequence of activated rat Her-2/neu gene overexpression is that a large, fast-growing lobular carcinoma is palpable at week 33 in all 10 glands of control BALB-neuT mice injected with saline only. Neither vaccination with ECD nor with ECD-IL1βna plasmids significantly counteracts this aggressive carcinogenesis, whereas in a less aggressive model of Her-2/neu carcinogenesis, vaccination with the same ECD vector used in the present study provided a significant protection[17] that was further increased by coinjection of the ECD plasmid with a bicistronic vector coding for IL-12.[18]

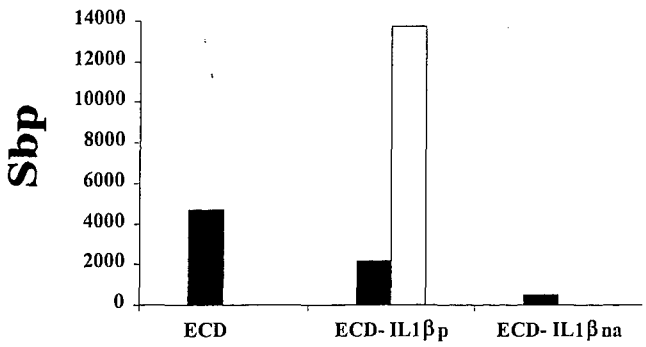

Figure 3 Presence of anti-p185^{neu} antibodies in the sera of variously immunized mice. Sera from mice immunized four times with ECD, ECD-IL1βp or ECD-IL1βna were collected at week 33, pooled and specific p185^{neu} Sbp was evaluated by flow cytometry after indirect immunofluorescence. Black columns, sera from mice displaying a palpable tumor in all 10 mammary glands, irrespective of vaccination regimen. White column, serum pool from mice vaccinated with ECD-IL1βp displaying 1–4 mammary tumors only.

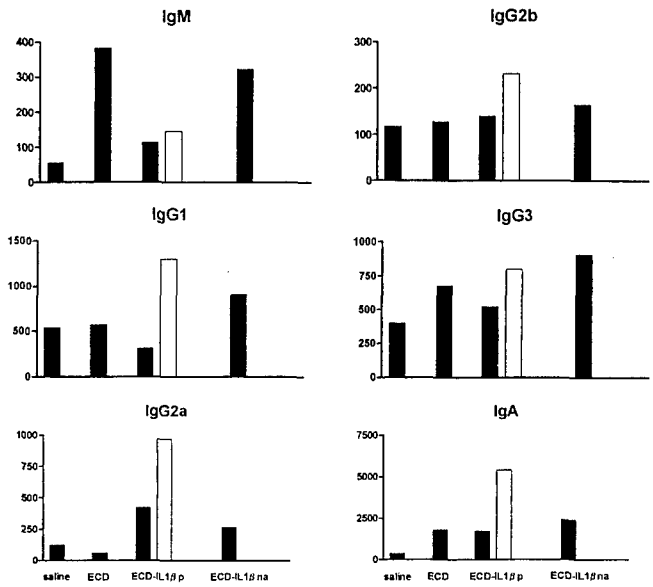

Figure 4 Distribution of immunoglobulin isotypes in the sera of immunized BALB-neuT mice. Serum pools from mice injected four times with saline only or immunized with ECD, ECD-IL1βp or ECD-IL1βna tested by radial immunodiffusion. Black columns, sera from mice displaying a palpable tumor in all 10 mammary glands, irrespective of vaccination regimen. White columns, serum pool from mice vaccinated with ECD-IL1βp displaying 1–4 mammary tumors only.

This critical immunomodulatory role played by IL1βp fits in well with our previous experience using this peptide as a systemic adjuvant.[12] Moreover, its sustained local presence in the tumor area activates an effective antitumor reaction against a poorly immunogenic transplantable mammary tumor.[12] Insertion of IL1βp augments the antitumor immune response induced by protein and DNA vaccines.[14] Moreover, vaccination with plasmids containing IL1βp DNA sequence[13,14] or protein antigen fused with IL1βp[13] increases the immunogenicity of many antigens.

In BALB-neuT mice, rat p185^{neu} is a self-protein already overexpressed by the mammary gland by the 3rd week of life.[16] Vaccination with ECD is unable to break this natural tolerance. Insertion of IL1βp DNA in the construct inhibits Her-2/neu carcinogenesis and is accompanied by lymphocyte infiltration of the stroma surrounding the TDLU and induction of anti-p185^{neu} antibodies. By contrast, no significant CTL response was found, despite all the *in vitro* restimulation attempts. This provocative finding fits in well with the absence of cytotoxicity we have found following vaccination of BALB/c and BALB-neuT mice against plasmids encoding both the ECD and TM portions of the p185^{neu}.[19] Even the presence of IL1βp signal is not enough to activate T killer cells against p185^{neu}.

The distribution of anti-p185^{neu} antibodies in the treatment groups also raises some puzzling issues. Only ECD-IL1βp immunized mice with evident inhibition of Her-2/neu carcinogenesis display a high anti-p185^{neu} antibody titer. While this may suggest a direct correlation

between antibody titer and inhibition, the low titer found in mice with large tumors may be due to antibody absorption by the $p185^{neu+}$ tumor cells or immunosuppression by such large tumors.

An issue not directly addressed in this paper but raised by the significant evidence in the literature is whether anti-$p185^{neu}$ antibodies induce a functional block of $p185^{neu}$ receptor function,[20] down-regulate its expression on the cell surface,[20,21] impede the formation of $p185^{neu}$ homo- or heterodimers that spontaneously transduce proliferative signals to the cells,[21,22] and block its ability to bind ligands.[23] These antibodies also significantly suppress the growth of transplantable $p185^{neu+}$ tumors,[24,25] the natural onset of mammary carcinomas in Her-2/neu transgenic mice,[21] and delay tumor growth in patients with Her-2/neu positive tumors.[26] A reduced r-$p185^{neu}$ expression could be sufficient to reverse their transformed phenotype.[20,21]

The marked increase in serum IgG2a and IgA in ECD-IL1βp-immunized mice suggests a more finely divided reaction scenario wherein leukocytes infiltrating the tumor site may play an important effector role.[27–29] These antibody isotypes activate PMN and other cells to mediate antibody dependent cellular cytotoxicity (ADCC)[25,30] and complement-dependent cytotoxicity (IgG2a), and inhibit the growth of $p185^{neu+}$ tumor *in vivo*.[25] IgG and IgA may synergistically promote ADCC by PMN and other leukocytes[25,31] that massively infiltrate hyperplastic lesions. It is also possible that the concentration of secretory IgA may reach particularly high levels in the mammary gland, where their inhibitory activity is required.

Material and methods

Mice

Inbred BALB-neuT mice overexpressing the transforming rat Her-2/neu oncogene ($neuT^+/neuT^-$) driven by the mouse mammary tumor virus promoter were produced and screened for the presence of the transgene as previously described in detail.[15] Groups of individually tagged virgin BALB-neuT females bred under specific pathogen-free conditions by Charles River, Calco, Italy were treated in accordance with European Union and institutional guidelines. Since all 10 mammary glands of BALB-neuT females naturally undergo carcinogenic transformation with a definite progression,[15] these were inspected weekly, and tumor masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, when mice were killed for humane reasons. Except where otherwise specified, surviving BALB-neuT mice were killed at 33 weeks.[15] As some immunized mice do not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas per mouse was calculated as cumulative number of incident tumors per total number of BALB-neuT mice.

DNA expression vectors and vaccination

The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA, USA) by deleting the SV40 promoter, neomycin resistance gene and SV40 polyA. The sequence for the extracellular domain of transforming mutated rat $p185^{neu}$ was generated from the PCR product using the primers 3′-CGCAAGCTTCATCATGGAGCTGGC-5′ and 3′-CGGAATTCGGGCTGGCTCTCTGCTC-5′ and the primers 3′-CGCAAGCTTCATGGAGCTGGC-5′ and 3′-ATGAATTCTTTCCGCATCGTGTACTTCTTCCGG-5′, respectively, as previously described.[17] PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI and cloned into the multiple cloning site of the pCMV plasmid in order to obtain the ECD plasmid used in this work. The immunomodulatory IL1βp nonapeptide WQGEESNDK corresponding to amino acids 163–171 and the control non-active IL1βna nonapeptide EGTEKDQVS corresponding to amino acids 189–197 of the human IL-1β were cloned in-frame into *Eco*RI and *Xba*I sites obtained by incorporating two complementary and overlapping phosphorylated oligonucleotides encoding each of the two peptides with *Eco*RI and *Xba*I sites: 5′-AATTCGGTTCAGGGTGAAGAAAGTAACGATAAATAAT-3′ (IL1βp-Forw) and 5′CTAGATTATTTATCGTTACTTTC TTCACCCTGAACCG-3′ (IL1βp-Rew); 5′-AATTCGGAAGGTACCGAAAAAGATCAGGTTAGTTAAT-3′ (IL1βna-Forw) and 5′-CTAGATTAACTAACCTGATCTTTTTCGGTACCTTCCG-3′ (IL1βna-Rew). *Escherichia coli* strain DH5 was transformed with ECD, ECD-IL1βp and ECD-IL1βna plasmids and then grown in Luria–Bertani medium (Sigma, St Louis, MO, USA) as previously described.[17] Large-scale preparation of the plasmids was carried out by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA, USA). DNA was then precipitated, suspended in sterile saline at the concentration of 1 mg/ml and stored in aliquots at –20°C for subsequent use in immunization protocols. Plasmids were injected (100 μg per injection) into the quadriceps muscle through a 28-gauge needle syringe. BALB-neuT mice were immunized at the 6th, 12th, 18th and 24th week.

Morphologic analysis

Groups of three BALB-neuT mice were killed at the indicated times. For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin or Giemsa.

Cell lines

TUBO and N202.1 cells are cloned cell lines established *in vitro* from lobular carcinomas that arose spontaneously in a BALB-neuT and in an FVB-neuN transgenic mouse, respectively.[19] N202.1A and N202.1E are two clones randomly derived from N202.1.[32] Cytofluorimetric analysis indicates that N202.1A expresses high levels of $p185^{neu}$, whereas this expression is not detectable in N202.1E.[32] Both clones were cultured in DMEM (BioWhittaker Europe, Verviers, Belgium) supplemented with 20% FBS (Life Technologies, San Giuliano Milanese, Italy).

Cytotoxicity assays

The cytotoxicity of lymphocytes from the mice in each group was tested immediately or after *in vitro* restimulation. Lymphocytes (1×10^7) were stimulated for 6 days with 5×10^5 irradiated TUBO cells as previously described.[33] To get better stimulation, this basic design

was variously changed in the several repeats of the test. Other rat Her-2/neu expressing BALB/c target cells were also used as stimulator and target cells. Moreover, the suppressor activity of stimulator rat Her-2/neu BALB/c cells was ruled out by adding progressive numbers of third-party TUBO cells in mixed lymphocyte and allogeneic target cell interactions as previously described.[34] Cytotoxicity of fresh and restimulated lymphocytes was assayed in 4 and 18 h ^{51}Cr release assays, 48 and 72 h [^{3}H]TdR release assays as previously described in detail.[33,34] In all these tests both TUBO cells and other rat Her-2/neu expressing BALB/c target cells were highly lysable by allogeneic cytotoxic T lymphocytes.

Cytofluorimetric evaluation of anti r-p185^{neu} antibodies

Sera of six BALB-neuT mice immunized with ECD, ECD-IL1βp and ECD-IL1βna were collected at 33 weeks when they all display 10 palpable tumors and pooled. The sera of ECD-IL1βp immunized mice displaying only 1–4 palpable tumors at 33 weeks was separately collected. The control sera were a pool from six mice injected with saline only. The ability of sera to bind r-p185^{neu} was evaluated by flow cytometry. 2×10^5 N02.1A or N202.1E cells from *in vitro* cultures, washed twice with cold PBS supplemented with 2% BSA and 0.05% sodium azide, were stained in a standard indirect immunofluorescence procedure with 50 μl of 1:10 dilution in PBS-azide-BSA of control or immune sera. A fluorescein-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) was used as second-step Ab. The cells were resuspended in PBS-azide-BSA containing 1 mg/ml of propidium iodide to gate out dead cells, and evaluated in a FACScan (Becton Dickinson). The specific N202.1A binding potential (Sbp) of the sera was calculated as follows: [(% positive cells with test serum) (fluorescence mean)] – [(% positive cells with control serum) (fluorescence mean)] X serum dilution, as previoulsy described in detail.[34] 5×10^3 viable cells were analyzed in each evaluation.

Serum concentration of Ig isotypes

The concentration of IgA, IgM, IgG1, IgG2a, IgG2b and IgG3a isotypes in pool of mice injected four times with saline only or immunized with ECD, ECD-IL1βp or ECD-IL1βna was determined by the radial immunodiffusion test (The Binding Site, Birmingham, UK).

Statistical analysis

Differences in tumor incidence were evaluated by the Mantel–Haenszel log-rank test, those in tumor/mouse numbers by Wilcoxon's rank sum test and those in the number of tumor infiltrating cells by Student's *t* test.

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Inhibition of Mammary Carcinogenesis by Systemic Interleukin 12 or p185^{neu} DNA Vaccination in Her-2/*neu* Transgenic BALB/c Mice[1]

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Abstract

Because BALB/c mice transgenic for the rat Her-2/*neu* oncogene develop multifocal carcinomas in all mammary glands by week 33, they constitute an aggressive model for investigation of treatments designed to oppose mammary carcinogenesis. Nonspecific immune reaction elicited by systemic interleukin (IL)-12 both delayed the appearance of the first tumor and reduced the number of glands affected. However, only 5% of mice were tumor free at week 33. On the other hand, specific vaccination with plasmids encoding for the rat p185^{neu} resulted in a further delay, so much so that 58% of mice were tumor free at week 33. No CTL response was evoked in either IL-12-treated or DNA-vaccinated mice, whereas an anti-rat p185^{neu} antibody response was evident in the latter. Pathological examinations showed that in both IL-12-treated and DNA-vaccinated mice, the tumor growth area was infiltrated by reactive cells associated with expression of endothelial adhesion molecules and antiangiogenic proinflammatory cytokines. In the vaccinated mice, reduction of the number of cells expressing rat p185^{neu} was combined with down-regulation of its membrane expression and even a marked inhibition in development of the terminal ductal lobular units. The reactive infiltrate in vaccinated mice contained numerous granulocytes that likely played an antiangiogenic and angiodestructive role and also joined other cells in the antibody-mediated killing of the r-p185^{neu+} cells. These results suggest that the elicitation of nonspecific and specific immunity could be beneficially used in individuals with a high risk of developing tumors.

Introduction

Breast cancer is the most frequent malignancy of women worldwide (1). The Her-2/*neu* oncogene is involved in human mammary carcinogenesis. Its amplification and overexpression, in fact, have been observed in a large percentage of primary human breast cancers (2–5). The generation of mouse strains transgenic for this oncogene has allowed *in vivo* evaluation of its role in the genesis and progression of a spontaneous murine mammary carcinoma (6–11). This model is of particular interest because it is composed of young, tumor-free mice that will inevitably develop cancer and as such are comparable with humans carrying a high genetic risk of cancer.

Immunotherapy with cytokines, or more specific management by means of vaccination, may be a reasonable option in the inhibition of carcinogenesis (12, 13). Both animal experiments and clinical trials have demonstrated the effectiveness of nonspecific and specific immunotherapy against residual disease and incipient metastases and in the control of recurrences (14, 15).

This report shows that treatment with systemic IL[3]-12 or DNA vaccination against the rat Her-2/*neu* gene product, r-p185^{neu}, opposes tumor growth in BALB/c mice transgenic for the transforming rat Her-2/*neu* oncogene (BALB-neuT). In these mice, r-p185^{neu} expression in the TDLUs results in the rapid and synchronous development of multifocal mammary tumors (11). The mechanisms by which these two approaches inhibit tumor growth were compared. In both treatments, inhibition was associated with deficient peri- and intratumoral angiogenesis, infiltration of reactive cells, production of proinflammatory cytokines, and iNOS activation. The greater efficacy of vaccination appears to stem from the production of anti-r-p185^{neu} Abs that oppose mammary proliferation and induces Ab-dependent cellular cytotoxicity against p185^{neu}-expressing mammary epithelial cells.

Materials and Methods

Mice. Inbred BALB-neuT mice overexpressing the transforming rat Her-2/*neu* oncogene (neuT$^+$/neuT$^-$) driven by the mouse mammary tumor virus promoter were produced and screened for the presence of the transgene, as described in detail previously (16). Groups of individually tagged virgin BALB-neuT females, bred under specific pathogen-free conditions by

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[3] The abbreviations used are: IL, interleukin; ECD-TM, extracellular and transmembrane; iNOS, nitric oxide synthase; MSA, mouse serum albumin; PCNA, proliferating cell nuclear antigen; sbp, specific binding potential; TDLU, terminal ductal lobular unit; Ab, antibody; TNF, tumor necrosis factor; NK, natural killer; MIP, macrophage inflammatory protein.

Charles River (Calco, Italy), were treated in accordance with European Union and institutional guidelines. Because all 10 mammary glands of BALB-neuT females undergo carcinogenic transformation with a definite progression (16), the glands were inspected weekly, and tumor masses were measured with calipers in two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, at which time mice were sacrificed for humane reasons. Except where otherwise specified, surviving BALB-neuT mice were sacrificed at 33 weeks (16). Because some treated mice did not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas/mouse was calculated as the cumulative number of incident tumors/number of tumor-bearing BALB-neuT mice.

IL-12 Administration. IL-12 (Genetics Institute, Cambridge, MA) in HBSS supplemented with 0.01% MSA (Sigma Chemical Co., St. Louis, MO) was administered i.p. Following previous indications (17), mice were given the most effective schedule IL-12 administration. It consisted of seven 5-day courses of MSA only (MSA controls) or MSA plus IL-12 administered for 2 consecutive weeks, followed by 2 weeks off starting at the second week of age and continuing on the 5th, 6th, 9th, 10th, 13th, and 14th weeks. The first course consisted of 50 ng of IL-12/day; the subsequent six courses was 100 ng of IL-12/day. Other groups of mice remained untreated. Because no appreciable differences in tumor growth rate and in pathological findings were found between the untreated and the MSA controls, only the data of the first group are shown.

DNA Expression Vectors and Vaccination. The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A). The sequences for the extracellular and transmembrane domain of mutated r-p185^{neu} were generated from the PCR product using the primers 3′-CGCAAGCTTCATGGAGCTGGC-5′ and 3′-ATGAATTCTTTCCGCATCGTGTACTTCTTCCGG-5′, as described previously (18). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the plasmid used in this work coding the extracellular and transmembrane domains of r-p185^{neu} (ECD-TM plasmids). The pcDNA3 plasmid (Invitrogen) was used as control (pcDNA3 plasmid). *Escherichia coli* strain DH5α was transformed with ECD-TM and pcDNA3 plasmids and then grown in Luria-Bertani medium (Sigma; Ref. 18). Large-scale preparation of the plasmids was carried out by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Inc., Chatsworth, CA). DNA was then precipitated, suspended in sterile saline at the concentration of 1 mg/ml, and stored in aliquots at −20°C for subsequent use in immunization protocols. Plasmids (100 μg/injection) were injected into the quadriceps muscle through a 28-gauge needle syringe. BALB-neuT mice were immunized at the 6th, 12th, 18th, and 24th weeks of age. Other groups of mice remained untreated. Because no appreciable differences in tumor growth rate and in pathological findings were found between the untreated and the pcDNA3 immunized mice, only the data of the latter group are shown.

Flow Cytometry. To evaluate the presence of Ab to p185^{neu}, serum pools were collected at 33 weeks of age from six control and six ECD-TM immunized BALB-neuT mice. The specific r-p185^{neu} binding potential (sbp) of the sera was evaluated by flow cytometry after indirect immunofluorescence (19) using the r-p185^{neu}-positive tumor cell line (N202.1A) established *in vitro* starting from a mammary carcinoma that arose in a FVB female mouse transgenic for the rat Her-2/*neu* (20). N202.1A cells (2×10^5), washed twice with cold PBS supplemented with 2% BSA and 0.05% sodium azide, were stained in a standard indirect immunofluorescence procedure with 50 μl of 1:10 dilution in PBS-azide-BSA of control or immune sera, followed by a fluorescein-conjugated rabbit antimouse immunoglobulin (Dako, Glostrup, Denmark) to evaluate the total sbp. The sbp of the various immunoglobulin isotypes were evaluated using biotin-conjugated goat antimouse immunoglobulin IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Caltag, Burlingame, CA). Labeling steps were followed by incubation for 30 min at 4°C, washes with cold PBS-azide-BSA, and incubation with FITC streptavidin (Dako) for 30 min at 4°C. Stained cells were analyzed with a FACScan flow cytometer, and the sbp/50 μl of serum was calculated as follows [(% positive cells with immune serum) (fluorescence mean)] − [positive cells with control serum) (fluorescence mean)] per serum dilution as described in detail previously (19).

Morphological Analysis. Groups of two to three BALB-neuT mice were sacrificed at the indicated times each week until the 33rd week. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E or Giemsa. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with anti-dendritic cells (NLDC 145; Cederlane), anti-CD4, anti-CD8a (both from Sera-Lab, Crawley Down, Sussex, United Kingdom), anti-Mac1 (anti-CD11b/CD18), anti-Mac3, and anti-Ia (all from Boehringer Mannheim, Milan, Italy), anti-granulocyte RB6-8C5, provided by Dr. R. L. Coffman (DNAX, Inc., Palo Alto, CA), anti-asialo GM1 (Wako Chemicals, Dusseldorf, Germany), anti-endothelial cells (mEC-13.324), and anti-ELAM1 (E selectin; both provided by Dr. A. Vecchi, Istituto M. Negri, Milano, Italy); anti-ICAM1 (CD54), anti-VCAM1 (PharMingen, San Diego, CA), anti-IL-4, anti-IL-6, anti-IL-10, anti-IL-12, and anti-MCP1 (PharMingen); anti-MIP2 (Walter Occhiena Srl, Torino, Italy); anti-RANTES (Peprotech, Inc., Rocky Hill, NJ); anti-IL-1β (Genzyme, Cambridge, MA), anti-TNF-α (Immuno Kontact, Frankfurt, Germany), anti-IFN-γ (provided by Dr. S. Landolfo, University of Turin, Torino, Italy), and anti-iNOS (Transduction Laboratories, Lexington, KY) Ab. To evaluate the expression of r-p185^{neu} antigen and PCNA, paraffin-embedded sections were tested with anti-neu Ab (C-18)-G (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-PCNA (Yelm, Roma, Italy) Ab. After washing, they were overlaid with biotinylated goat antirat, antihamster, and antirabbit or horse antigoat immunoglobulin (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ab was removed by washing, and the slides were incubated with ABC complex/AP (Dako). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blinded fashion. From mice with multiple tumors, one sample/tumor growth area and 10 randomly chosen fields in each

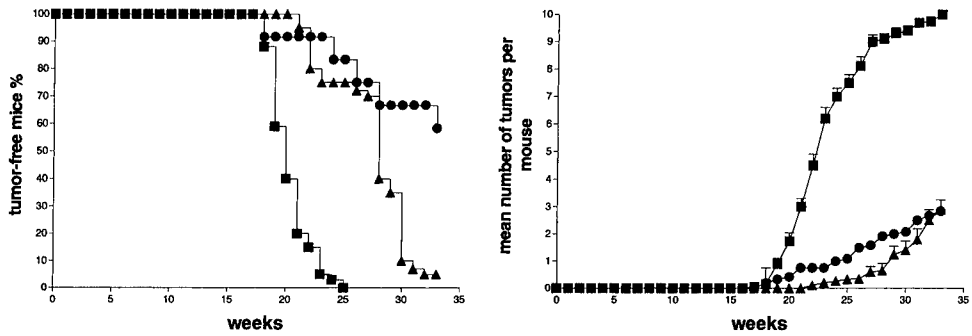

Fig. 1 Effect of systemic IL-12 and DNA vaccination on mammary carcinogenesis in BALB-neuT mice. The time of appearance of the first tumor (*left panel*) and mean number of palpable mammary carcinomas/mouse (*right panel*) in the group of 40 untreated mice (■), 20 mice treated with IL-12 (▲), and 12 mice immunized with ECD-TM plasmid (●) are shown. The mean number of palpable mammary carcinomas/mouse was calculated as the cumulative number of incident tumors/number of tumor-bearing BALB-neuT mice. At week 33, untreated controls display all (400 of 400) mammary glands with palpable carcinomas. The subgroup of tumor-bearing IL-12-treated mice display 34 of 50 (68%) palpable carcinomas, whereas the subgroup of tumor-bearing, plasmid-vaccinated mice display 57 of 190 (30%). The difference between these two subgroup is highly significant ($P < 0.0001$).

sample were evaluated for each point determination. Positive cells were counted under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2/field). The expression of adhesion molecules, cytokines, and mediators was defined as absent (−), scarcely (+/−), moderately (+), and frequently (++) present on cryostat sections tested with the corresponding Ab.

Cellular Cytotoxicity. CTLs were generated by culturing 10^7 responder spleen cells and 5×10^5 mitomycin C stimulator tumor cells for 6 days. Cytotoxicity was evaluated in a 4-h ^{51}Cr sodium chromate release assay by mixing in triplicate various concentrations of effector lymphocytes with 5×10^3 labeled target cells at 50:1, 25:1, 12:1, and 6:1 E:T ratios as described in detail previously (21).

Statistical Analysis. Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test; differences in tumor/mouse numbers were evaluated by χ^2 tests, and differences in the number of tumor-infiltrating cells were evaluated by Student's *t* test.

Results

As described previously, mammary carcinogenesis progresses in BALB-neuT mice in a way similar to that of lobular carcinoma in humans (11). Forty % of these mice displayed one or two palpable tumors at 20 weeks of age, whereas nearly 100% displayed multiple tumors in almost all of their mammary glands by the 27th week. As described previously (16), systemic IL-12 significantly delayed the appearance of the initial masses and markedly reduced the number of affected glands (Fig. 1*a*). However, at 33 weeks, only 5% of mice were free of palpable tumors (Fig. 1*b*). DNA vaccination resulted in a further delay, so much so that 58% of mice were tumor-free after 33 weeks and maintained this status after even 38 weeks. Although vaccination is significantly superior in affording complete protection, the number of glands affected (34 of 50) in the subgroup of vaccinated tumor-bearing mice was significantly higher than that observed in the subgroup of IL-12-treated, tumor-bearing mice (57 of 190).

Elicitation of an antitumor immune reactivity was assessed by looking for the induction of a CTL response in spc from untreated, IL-12 treated, and plasmid-vaccinated mice, both fresh and after *in vitro* restimulation, and by measuring the presence of anti-r-$p185^{neu}$ Ab in the sera. No CTL response was ever observed after both IL-12 and plasmid vaccination (data not shown). By contrast, a distinct Ab response was evident in the sera from the vaccinated mice and not in those from mice receiving IL-12 (Table 1). IgG2a, IgA, and IgG3 were the prominent isotypes.

Table 1 r-$p185^{neu+}$ N202.1A-labeled cells from sera of untreated control and ECD-TM-vaccinated BALB-neuT mice

	sbp × 10^3/ml of immune serum[a]					
Total Ig	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM
22.735	3.058	44.854	<1	26.296	35.827	<1

[a] Sera were collected at 33 weeks of age from six tumor-bearing untreated and six tumor-free ECD-TM-vaccinated BALB-neuT mice. Dead cells were gated on the basis of forward and side scatter. Viable cells (1×10^4) were analyzed in each evaluation. Values of sera from untreated mice were: total Ig, 2.49% of positive cells/mean, 17.47 of fluorescence intensity; IgA, 2.65%/mean, 434.12; IgM, 14.3%/mean, 221.46; IgG1, 2.91%/mean, 417.91; IgG2a, 1.26%/mean, 39.74; IgG2b, 1.43%/mean, 266.02; and IgG3, 1.27%/mean, 707.69.

The morphological features that correlated with the impaired progression of carcinogenesis were assessed by pathological comparison of the mammary glands from untreated, IL-12 treated, and vaccinated mice. Foci of atypical hyperplasia were already evident in some TDLUs of the untreated mice by the 3rd week. These then extended to most TDLUs and progressed to carcinoma *in situ* around the 15th week and invasive lobular carcinoma from the 20th week onward (Fig. 2*a*). These foci were also present at the 3rd week in the IL-12-treated mice but grew less extensively. The onset of carcinoma *in situ* was delayed and necrotic-hemorrhagic areas were visible in some mammary glands. A substantial reactive infiltrate was evident in the stroma around the limited hyperplastic (Fig. 2*b*), and neoplastic lesions were observed after the 25th week of age.

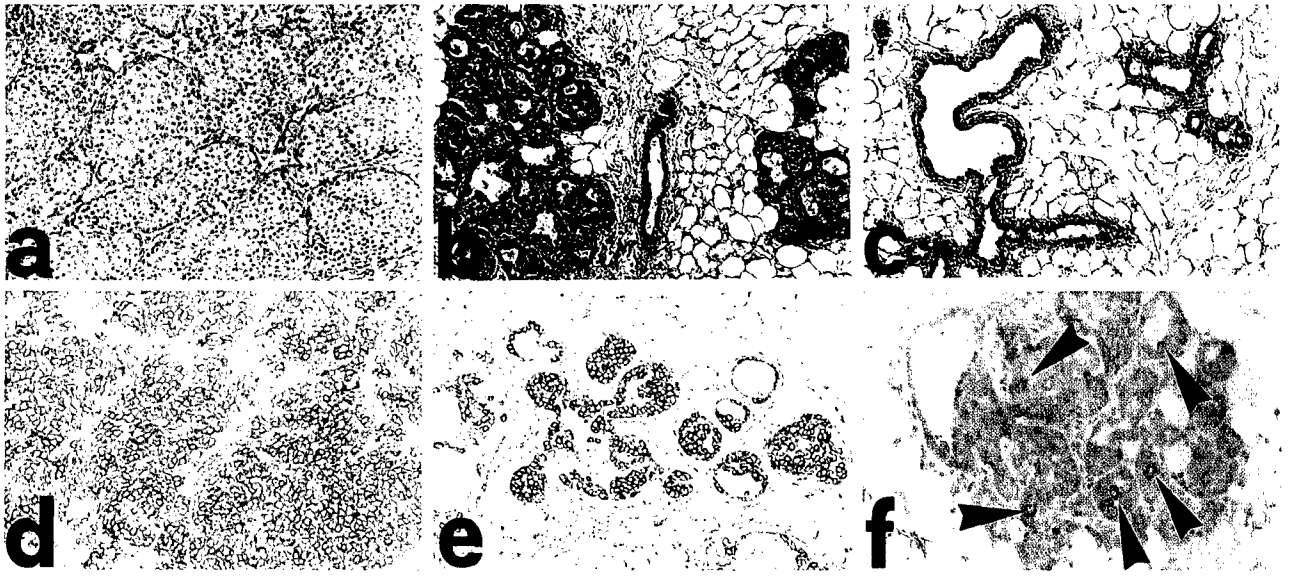

Fig. 2 Histology (*a–c*) and immunohistochemistry with anti-r-p185^{neu} Ab (*d–f*) of mammary tissue from untreated (*a* and *d*), IL-12 treated (*b* and *e*), and DNA-vaccinated (*c–f*) BALB-neuT mice at 27 weeks of age. An invasive lobular carcinoma with an alveolar arrangement spontaneously arising in one untreated mouse is shown in *a*. All of the carcinomatous cells express the r-p185^{neu} in the cytoplasm and on their membranes (*d*). Multiple foci of atypical hyperplasia characterized by ducts and lobules almost completely occupied by round epithelial cells assuming a stratified appearance are present in an IL-12-treated mouse (*b*). The hyperplastic cells exhibit both cytoplasmic and membrane r-p185 ^{neu} expression (*e*). The mammary glands of a vaccinated mouse (*c*) are mainly composed of ductules lined by a single layer of epithelial cells, and the development of TDLUs is completely blocked or markedly inhibited. A weakly developed hyperplastic area from the mammary tissue of a vaccinated mouse shows r-p185^{neu} expression mainly confined in the epithelial cell cytoplasm (*f*). A few cells with both membrane and cytoplasm expression (*arrowheads*) are mainly located in the innermost portion of the TDLU.

In the DNA-vaccinated mice, the picture was much the same as in the untreated mice until the 10th to 11th week. At the 15th week, after the second vaccination, the hyperplastic foci were less developed and less distributed. They were also surrounded by a distinct inflammatory infiltrate. There were no *in situ* carcinomas. At the 27th week, after the fourth vaccination, >40% of mice were free from both *in situ* and invasive tumors. The mammary glands were mainly composed of ductules lined with a single layer of epithelial cells (Fig. 2*c*), the development of TDLUs being completely blocked or markedly inhibited. Hyperplastic foci surrounded by reactive cells were occasionally found. Pathologically detectable lobular carcinomas were present in <60% of these mice, and their development was manifest in a mammary tissue absolutely less florid than that visible in the untreated mice. A distinct reactive infiltrate was noted in the peripheral stroma and especially in the hyperplastic areas on the edges of the tumors.

Immunohistochemical examination showed that this infiltrate was composed of various subsets of reactive cells (Table 2), mainly macrophages, granulocytes (Fig. 3*c*), and CD8+ lymphocytes. Their number was greater than in the untreated mice, as well as in those treated with IL-12. However, the latter displayed a significantly more marked infiltrate than untreated mice. The number of dendritic and NK cells in the lesions of mice treated with IL-12 or vaccinated was significantly higher than in the untreated mice.

The greater infiltrate in the IL-12-treated and vaccinated mice was associated with induction or up-regulation of endothelial adhesion molecules. A more conspicuous presence of granulocytes in the vaccinated mice was accompanied by a greater expression of ELAM1.

Reactivity toward the hyperplastic and/or neoplastic lesions in the IL-12-treated and vaccinated mice was also manifested by the local expression of proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) and chemokines chemotactic for macrophages (MCP1), granulocytes (MIP2; Fig. 3, *d–f*), and lymphocytes (IP10 and MIG) and mediators such as iNOS. The more marked expression of IP10 and MIG in the IL-12-treated and vaccinated mice was accompanied by reduced development of the vascular network, as assessed by the microvessel count (Table 2 and Fig. 3, *g–i*). In addition, many of the few vessels in the vaccinated mice were occluded by thrombi, and the continuity of their endothelium was interrupted (Fig. 3*l*; as assessed with the anti-CD31 Ab). Expression of vascular endothelial growth factor and basic fibroblast growth factor, the two main angiogenic factors, was similar in the treated and untreated mice.

Immunohistochemical evaluation of r-p185^{neu} expression and the proliferation rate of the epithelial cells showed that by the 27th week, nearly the whole of the mammary tissue of the untreated mice was occupied by hyperplastic or carcinomatous lesions whose cells highly expressed r-p185^{neu} in their cytoplasms and on their membranes (Fig. 3*d*). High positivity for PCNA corresponded to this picture and was more evident and frequent in the basal layer cells. Both membrane and cytoplasmic r-p185 ^{neu} positivity were apparent in the epithelial cells of the mammary lesions of the IL-12-treated mice (Fig. 2*e*). PCNA expression seemed to correlate with that of membrane

Table 2 Reactive cell content, expression of endothelial adhesion molecules, production of cytokines and mediators, and microvessel count in mammary glands of untreated and treated female BALB-neuT mice

Treatment	MSA[a]	IL-12[a]	Vaccine[a]
Reactive cells[b]			
Dendritic cells	1.7 ± 0.9	13.2 ± 2.7[c]	11.6 ± 3.1[c]
Macrophages	9.2 ± 4.0	12.3 ± 3.6	23.4 ± 4.2[d]
PMN	5.1 ± 1.8	7.4 ± 2.9	22.1 ± 6.2[d]
$CD8^+$ lymphocytes	2.0 ± 0.7	13.9 ± 4.3[c]	25.6 ± 5.9[d]
$CD4^+$ lymphocytes	1.7 ± 0.9	9.1 ± 3.8[c]	12.6 ± 4.7[c]
NK cells	3.2 ± 0.8	16.9 ± 4.7[c]	12.3 ± 4.1[c]
Endothelial adhesion molecules[c]			
ELAM-1	−	+/−	++
ICAM-1	+	+	++
VCAM-1	−	++	+
Cytokines and mediators			
IL-1β	−	+	+
TNF-α	+/−	+	++
IFN-γ	−	+	+
MCP-1	−	+	+/−
MIP-2	+/−	+/−	++
IP10	+	++	++
MIG	+	++	++
iNOS	−	++	++
Microvessel count	16.1 ± 3.3	9.1 ± 2.3[c]	7.3 ± 1.8[c]

[a] Analysis was performed in MSA-treated, IL-12-treated, and vaccinated 27-week-old mice. Breast tissue was mainly affected by carcinoma in MSA-treated mice and by atypical hyperplasia in IL-12-treated mice and in vaccinated mice.

[b] Cell and microvessel counts were performed at ×400 in 10 0.180-mm^2 randomly chosen fields/sample. Results are mean ± SD of positive cell/field evaluated on cryostat sections by immunohistochemistry.

[c,d] Values significantly different ($P < 0.001$) from corresponding values in: [c] MSA-treated mice; or [d] IL-12-treated mice.

[e] The expression of adhesion molecules, cytokines and mediators was defined as absent (−), scarcely (+/−), moderately (+), or strongly (++) present on cryostat sections prepared with the Ab.

r-$p185^{neu}$, although it was more evident in the basal layer cells, as in the untreated mice. The picture presented by these antigens in the vaccinated mice was very different. Most of their mammary tissue was composed of poorly branched ductules devoid of TDLUs and lined with non-r-$p185^{neu}$-expressing epithelial cells. In the weakly developed hyperplastic areas, r-$p185^{neu}$ expression was mainly cytoplasmic, and the few cells with both membrane and cytoplasmic expression were mainly located in the innermost portion of the ductal lobular structure and hence more distant from the basal membrane and the stroma (Fig. 2*f*). This immunohistochemical picture was only seen in the more peripheral portions of the carcinomas. Both membrane and cytoplasmic expression, in fact, were evident in their central portion. Low membrane r-$p185^{neu}$ positivity was accompanied by low PCNA positivity.

Discussion

The progression of Her-2/*neu* carcinogenesis that naturally occurs in BALB-neuT mice is significantly delayed by both systemic IL-12 treatment and DNA vaccination. However, DNA vaccination is more effective because ~60% of the vaccinated mice were tumor free at the 33th week, when all of the untreated and 95% of IL-12-treated mice displayed tumors.

Previous data suggest that systemic IL-12 effectively inhibits neoplastic proliferation by triggering nonspecific and inflammatory mechanisms (14, 22). IL-12 induces the production of IFN-γ and TNF-α by NK cells, and activated T cells promote the activation of the various reactive cell subsets that normally infiltrate the hyperplastic/tumor growth areas (16, 22). A major role was apparently played by NK cells, activated T cells, and intratumoral macrophages (14). The presence of cytokines secreted by the activated leukocytes infiltrating the hyperplastic/tumor growth area, as demonstrated immunohistochemically, leads to expression in the tumor vessels of adhesion molecules that guide additional leukocyte recruitment. The inflammatory infiltrate may thus be supposed to give rise to a proinflammatory cytokine cascade, which appears to be mainly oriented in the antiangiogenic direction (IP10 and MIG; Refs. 23 and 24). The weak development of hyperplastic foci is probably determined by the halting development of angiogenesis, as shown by the fewer vessels than in the untreated mice. The marked slowing down of tumor progression induced by IL-12 may thus be supposed to be based on induction of secondary and tertiary downstream mediators endowed with significant antiangiogenic activity as well as direct cytotoxic and cytolytic functions (25, 26).

The nonspecific reaction mechanisms activated by IL-12 acquire selectivity because IL-12 treatment induces the recruitment of many reactive cells in the tumor growth area (14, 22) and because it has been shown that mammary cells need a generous blood supply during the hyperplastic stage (11). The greater effectiveness of early treatment seems to rest on the ability of the reaction induced by IL-12 to inhibit the early epithelial proliferation, which results in hyperplasia (17). An explanation may thus be found for the observation that administration of IL-12 after the establishment of a carcinoma has virtually no effect (17). At this stage, the tumor's blood supply is assured by large, well-developed, and mature vessels against which the mediators induced by IL-12 do not appear to act. The notion that the antitumor activity of IL-12 mainly rests on nonspecific mechanisms is corroborated by the absence of CTLs and Abs against the tumor cells, despite the presence of many dendritic cells and lymphocytes in the tumor growth area.

The antitumor activity that follows vaccination apparently involves mechanisms that are morphologically similar to those induced by IL-12. In the tumor area and particularly in the hyperplasia stage, there is a marked infiltrate of reactive cells coupled with the expression of endothelial adhesion molecules and proinflammatory cytokines, including some with an efficacious antiangiogenic function, and iNOS activation. These events may be supposed to be the outcome of a reactivity specifically guided by anti-r-$p185^{neu}$ Abs.

Histological and immunohistochemical analysis reveals morphological changes ascribable to anti-r-$p185^{neu}$ Abs. After the first two vaccinations, the hyperplastic areas are less florid and surrounded by a marked inflammatory infiltrate. In addition, after four vaccinations r-$p185^{neu}$-expressing cells are fewer in number, and r-$p185^{neu}$ expression is mainly confined to the cytoplasm. Its expression on the cell membrane is typical of cells further from the stroma and hence from the vessels, the

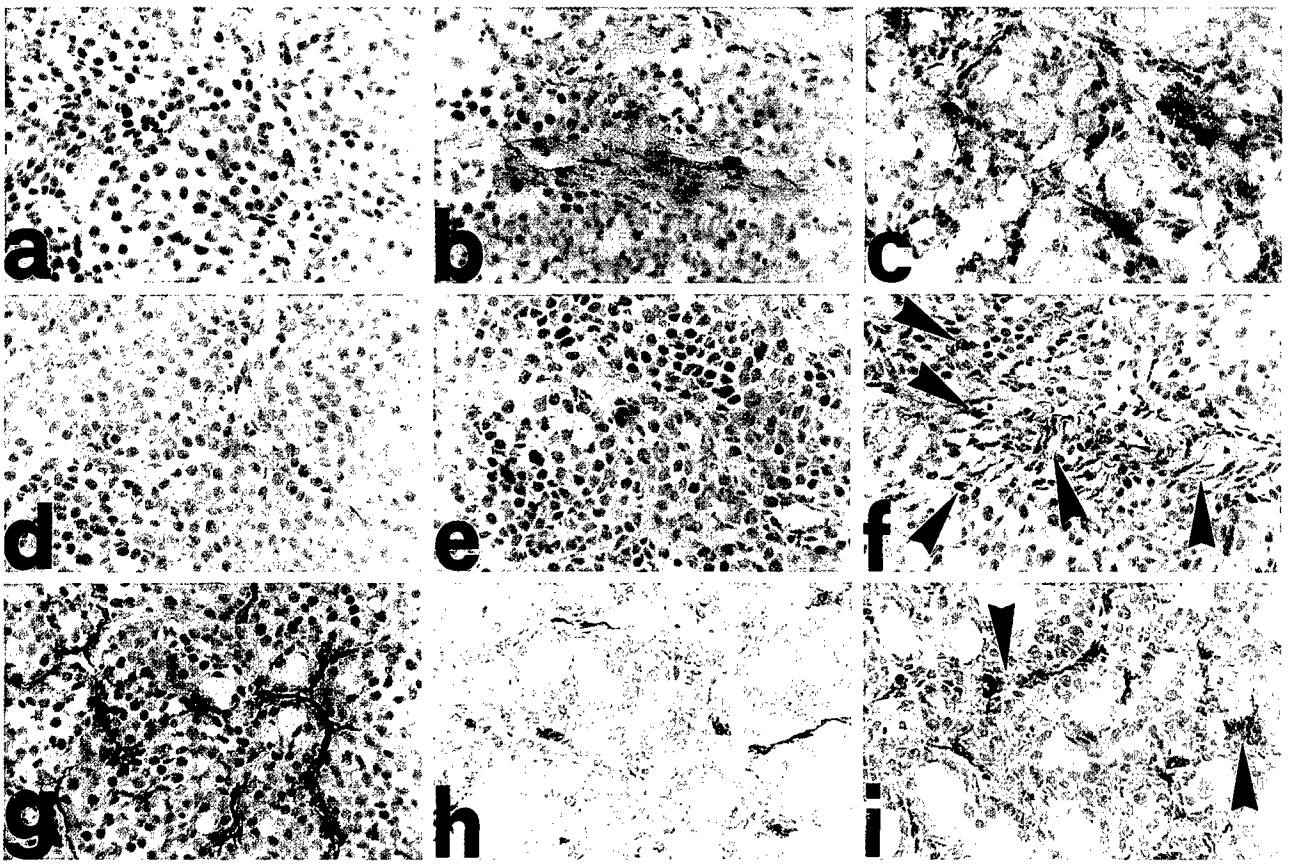

Fig. 3 Immunohistochemistry performed with an anti-granulocyte (*a–c*), anti-MIP2 (*d–f*), and anti-endothelial cell (*g–i*) Abs on untreated (*a, d,* and *g*), IL-12-treated (*b, c,* and *h*) and DNA-vaccinated (*c, f,* and *i*) mice. Granulocytes, scarcely present in the mammary lesions of untreated (*a*) and IL-12-treated (*b*) mice, are evident in the mammary tissue of vaccinated mice (*c*). MIP2 is expressed by endothelial and reactive cells (*arrowheads*) of mammary tissue of vaccinated mice (*f*), whereas its presence is scarce or absent in untreated (*d*) or IL-12-treated (*e*) mice. The anti-endothelial staining shows that numerous blood vessels are present in the delicate stroma of the lobular carcinoma that developed in an untreated mouse (*g*). Their number is reduced in hyperplastic lesions of IL-12-treated (*h*) and vaccinated (*i*) mice. In the latter, the continuity of the endothelium is sometimes interrupted (*arrowheads*).

reactive cells, and a possible Ab transudation. It is thus likely that the specific Abs both down-regulate membrane expression and impede transduction of the proliferative signals to the cell by functional blockade of its receptor (27–30). Although Her-2/*neu* overexpression is associated with elevated tumorigenicity, its down-regulation causes suppression of the cell-transforming phenotype induced by this oncogene (31). In tumor-bearing vaccinated animals, the morphological picture suggests that this Ab and reactive action is limited to the peripheral, more accessible layers of the tumor, whereas all of the cells in its central portions express r-p185^{neu} on their membranes, and the delicate stroma does not display any reactive infiltrate. In the vaccinated animals still tumor free after 27–30 weeks, anti-r-p185^{neu} reactivity is so intense that the mammary tissue is mostly formed of ductules almost devoid of TDLUs, amputated of their portions where p185^{neu} is normally expressed, and hence unable to proliferate. In this way, vaccination appears to cause a selective mastectomy, removing a structure of critical importance in the development of mammary lobules and thus of lobular carcinomas.

In the reactive infiltrate around the lesions of vaccinated mice, numerous granulocytes probably attracted by the local high MIP2 production (32, 33) were found. The function of these cells, mainly considered on account of their nonspecific role in inflammation, must be reassessed in the light of numerous observations suggesting that they participate in the antitumor reactivity. Several experimental models have shown that the presence of granulocytes in the tumor growth area is accompanied by tumor destruction and that this does not take place in granulocyte-depleted animals. In addition to destroying the vessel network (34, 35), Ab-guided granulocytes are probably antiangiogenic through their production of IP10 and MIG (36, 37). This function could underlie the endothelial damage seen in the poorly developed vessel network of the vaccinated animals. In this case, the nonspecific cytokine cascade, together with the antiangiogenic activity, are both triggered by specific anti-r-p185^{neu} Abs that form complement-activating immunocomplexes and guide Fc receptor-positive leukocytes into the tumor. Granulocytes may also collaborate with other cells in the Ab-dependent cellular cytotoxicity-mediated killing (38, 39) of r-p185^{neu}-expressing cells. These considerations indicate that granulocytes may be regarded as an extremely efficacious ef-

fector arm, even in a specific antitumor response elicited by vaccination with a tumor antigen.

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Ability of Systemic Interleukin-12 to Hamper Progressive Stages of Mammary Carcinogenesis in HER2/*neu* Transgenic Mice[1]

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ABSTRACT

Previous studies in mice have shown that chronic administration of recombinant interleukin-12 (IL-12) hampers the progression of both chemical- and oncogene-dependent carcinogenesis. This suggests that a new preventive strategy may be envisaged for individuals with a genetic risk of cancer or carrying preneoplastic lesions. Starting at progressive stages of mammary carcinogenesis, female BALB/c and FVB mice carrying the activated rat HER2/*neu* oncogene (BALB-neuT) or the proto-oncogene (FVB-neuN) under the mouse mammary tumor virus promoter received multiple 5-day courses of different doses of IL-12. The times of tumor appearance, multiplicity, and histopathological features of the neoplastic lesions were evaluated. In both BALB-neuT and FVB-neuN mice, 5-day i.p. courses of 50/100 ng of IL-12/day inhibited mammary carcinogenesis when they coincided with the progression of early preneoplastic lesions. Inhibition appears to depend primarily on the ability of IL-12 to interfere with early tumor angiogenesis. Later treatments are much less effective, and daily doses of 10 and 2 ng are useless. The efficacy of early IL-12 courses suggests that they could be used to prevent mammary tumors in individuals at risk, whereas their lower efficacy in later stages of carcinogenesis and the dose range required pose some constraints on their use in the management of overt preneoplastic lesions. Precise understanding of tumor progression means that effective treatments can be commenced relatively late in the life of individuals at risk and that no lifetime administration is required.

INTRODUCTION

The remarkable ability of systematically injected recombinant IL-12[3] to inhibit transplantable mouse tumors (1–6) appears to rest on its induction of IFN-γ (2, 4), tumor necrosis factor α (5), and granulocyte/macrophage colony-stimulating factor (6). These secondary cytokines then induce other downstream factors that trigger a complex antitumor reaction. By acting on the endothelial cells of newly formed vessels, these mediators inhibit tumor neoangiogenesis (7, 8), induce the expression of adhesion molecules, and recruit leukocytes at the tumor site (7, 9). They also favor the elicitation of cytolytic effector cells and antitumor antibodies (3, 7, 10–12), whereas their presence in the tumor microenvironment affects tumor cells directly by inducing the overexpression of MHC glycoproteins (13) and switching the production of angiogenic factors to that of antiangiogenic factors (14).

IL-12 also hampers the progression of both chemical-(15) and *neu* oncogene-dependent (16) carcinogenesis and would thus seem open to exploitation as a preventive agent (17) because genetic screening is singling out individuals with a defined genetic risk of cancer (18), and preneoplastic lesions are being detected by early diagnosis programs (19).

To determine the stage of mammary carcinogenesis in which IL-12 most successfully inhibits the progression of preneoplastic lesions into invasive tumors, we used females of two transgenic mouse strains expressing the rat HER2/*neu* oncogene in the mammary gland. Although temporally differentiated by their kinetics, these two models of progression through atypical hyperplasia to *in situ* carcinoma and invasive carcinomas closely reproduce a few features of mammary carcinogenesis in women (16).

MATERIALS AND METHODS

Mice. BALB/c mice overexpressing the activated rat HER2/*neu* oncogene driven by the mouse mammary tumor virus (MMTV) promoter (Ref. 20; BALB-neuT) in their mammary glands were bred in our animal facilities (for details, see Ref. 16). A colony of FVB mice (N#202) carrying the rat HER2/*neu* proto-oncogene driven by the MMTV promoter (Ref. 21; FVB-neuN) was maintained under strict inbreeding from breeding pairs obtained from Dr. W. J. Muller (McMaster University, Hamilton, Ontario, Canada) as described previously (16). Groups of individually tagged virgin females were used. Their mammary glands were inspected weekly, and tumor masses were measured with calipers in two perpendicular diameters (16). Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored weekly until all 10 mammary glands displayed a palpable tumor or until one tumor exceeded an average diameter of 1.5 cm, at which time mice were sacrificed for humane reasons. Surviving BALB-neuT mice were sacrificed at the 33rd week, when tumor masses were evident in all 10 mammary glands; FVB-neuN mice were sacrificed at 90 weeks, when they displayed a mean number of 2.5 tumors/mouse.

IL-12 Administration. IL-12 (Genetics Institute, Cambridge, MA) in HBSS supplemented with 0.01% MSA (Sigma, St. Louis, MO) was administered i.p. At the times indicated, mice received seven 5-day courses of MSA only (MSA controls) or MSA plus IL-12. Other groups of mice remained untreated. Because no appreciable differences in tumor growth rate and pathological findings were found between the untreated mice and the MSA controls, only the data of the latter group are shown. The first course consisted of 50 ng of IL-12/day, and the subsequent six courses consisted of 100 ng of IL-12/day. These seven courses were administered at different times (Fig. 1). BALB-neuT mice assigned to the chronic treatment group received the first course at the 2nd week of age. From the 5th to the 25th week, courses were repeated every 4th week. Mice assigned to the late treatment group received the courses from the 13th to the 25th week. They were treated for 2 consecutive weeks, followed by 2 weeks off. Mice in the early treatment group received IL-12 beginning at the 2nd week and ending at week 14. In a few experiments, the early treatment was also performed with 10 and 2 ng in all seven courses. FVB-neuN mice received the courses every 4th week, starting on the 6th (6-week-old treatment), 22nd (22-week-old treatment), or 28th (28-week-old treatment) week of age. All of these treatments continued until week 90.

Histological and Immunohistochemical Analysis. Groups of three IL-12-treated and untreated BALB-neuT mice were killed at 15, 25, and 30 weeks of age, whereas similar groups of FVB-neuN mice were sacrificed at weeks 15, 20, 22, 25, 27, and 30. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm,

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[3] The abbreviations used are: IL, interleukin; MSA, mouse serum albumin; MMTV, mouse mammary tumor virus; PCNA, proliferating cell nuclear antigen.

and stained with H&E or Giemsa. For immunohistochemistry, formalin-fixed, paraffin-embedded, or acetone-fixed cryostat sections were incubated for 30 min with antiendothelial cells (mEC-13.324; Ref. 22) and PCNA (Ylem, Rome, Italy) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat antirat and mouse antigoat IgG (Vector Laboratories, Burlingame, CA) for 30 min. Unbound antibodies were removed by washing, and the slides were incubated with avidin-biotin complex/alkaline phosphatase (DAKO, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blind fashion. Two or more samples (one per tumor growth area) and 10 randomly chosen fields in each sample from mice with multiple hyperplastic foci or tumors were evaluated for each determination. Individual microvessels were counted under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2 per field). The rate of immunoreactivity for PCNA was obtained by counting the number of positive cells/number of total cells in the ductular and lobular structures under a microscope ×600 field (×60 objective and ×10 ocular lens; 0.120 mm^2 per field).

Statistical Analysis. Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test; differences in tumor/mouse numbers, the number of microvessels, and PCNA immunoreactive cells were evaluated by Student's *t* test.

RESULTS

IL-12 Delay of Carcinogenesis in BALB-neuT Mice. With a slightly asynchronous but consistent pattern, all mammary glands of untreated and MSA control BALB-neuT female mice progress into invasive carcinoma (Fig. 1; Ref. 16). Atypical hyperplasia of small lobular ducts and lobules is already evident at the 2nd week of age. At the 10th week, proliferating epithelial cells occlude the ductules and acini within the lobules. Vigorous capillary proliferation is evident at the 15th week, when atypical hyperplasia is prominent, often assuming the aspect of carcinoma *in situ* (Fig. 2*a*). Near the 20th week, the neoplastic ductular-lobular structures progressively expand and invade the surrounding tissues, and at least one palpable tumor mass is detectable around the 19th week (Fig. 3, *bottom panel*). Invasive lobular carcinomas (Fig. 4*a*) develop progressively, and at the 33rd week, tumor masses are palpable in all 10 mammary glands.

To evaluate the ability of IL-12 to inhibit this progression, mice received seven 5-day courses of IL-12 at different times (Fig. 1). In the chronic treatment, the courses started in the 2nd week and continued until the 25th week. Both a delay in the onset of the first mammary tumor and a 50% reduction in the number of mammary glands with a palpable tumor at 33 weeks (when the experiment was ended) were observed as compared with MSA controls (Fig. 3). To assess whether IL-12 is also effective during later phases, other 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 did reduced the number of tumors at week 33 by 22%. The early treatment began at the 2nd week and continued until week 14. The delay in onset of the first tumor and the reduction in the number of tumors are significantly higher than those seen in the chronic treatment group. When the early treatment was further split into shorter 4-week administration schedules, much less protection was observed (data not shown).

Pathology of Mammary Lesions in BALB-neuT Mice. A similarly widespread atypical hyperplasia of small lobular ducts and lobules with multiple foci of carcinoma *in situ* was evident at week 15 in the MSA controls and in the late treatment group that had received two IL-12 courses only at that time. However, in the latter group distinct vascular damage associated with few reactive cells close to hyperplastic and neoplastic lobules was evident. Mice from the chronic and early treatment groups revealed a less widely distributed

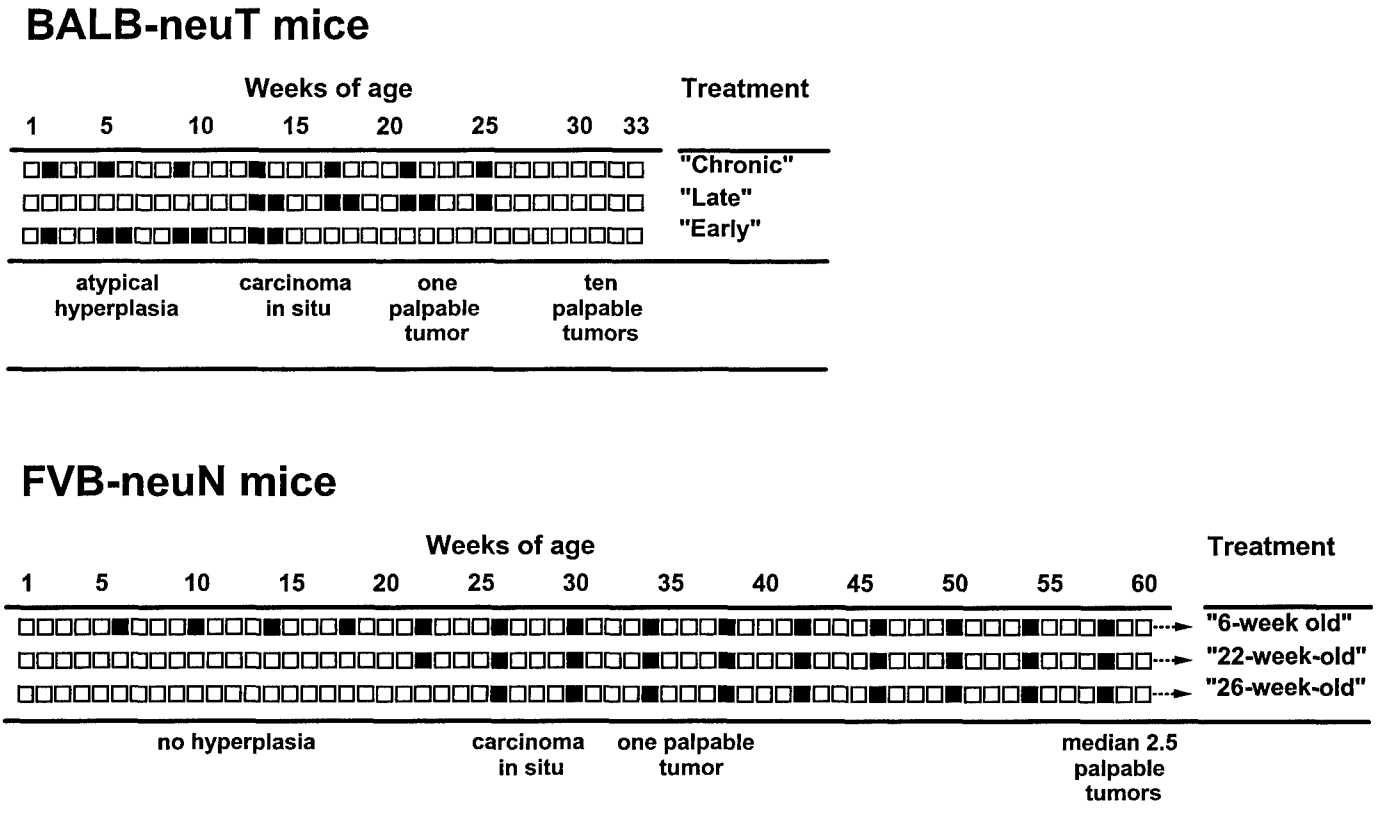

Fig. 1. Treatment outline. ■, weeks in which mice received 5-day courses (Monday through Friday) of daily i.p. injections of IL-12 or MSA only during the progression of HER2/*neu* mammary carcinogenesis.

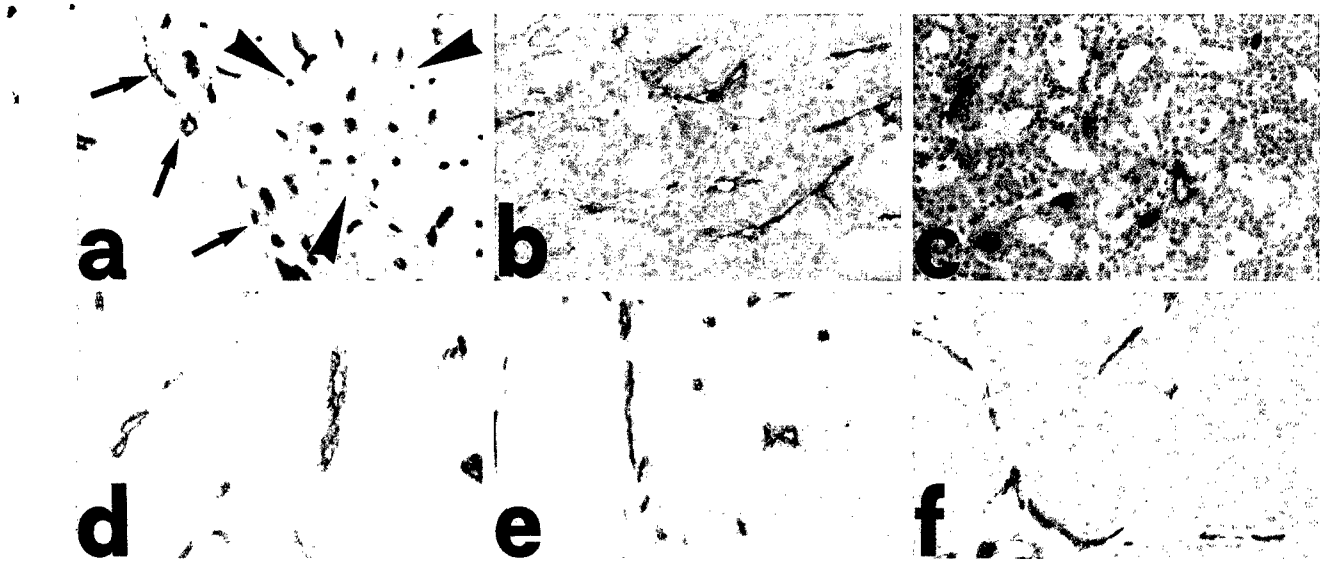

Fig. 2. Vascularization of mammary lesions in BALB-neuT mice. At 15 weeks, mammary glands from MSA control mice display numerous capillary sprouts (*arrowheads*) inside atypical hyperplastic areas, whereas scanty vascularization (*arrows*) is present at the periphery of *in situ* carcinoma (*a*). In hyperplastic mammary tissue from mice in the chronic treatment group, a clear reduction in the number of microvessels is evident (*b*). Mice from the early treatment group (*c*) display a marked reduction associated with a defective vascular network. At 25 weeks of age, the differences in the vascular architecture of the neoplastic lesions from the MSA control mice (*d*), chronic (*e*), and early (*f*) treatment groups are less evident.

atypical hyperplasia. Rare foci of carcinoma *in situ* were present in tissues from mice of the chronic treatment group, but not in those from the early treatment group (data not shown). At week 25, invasive carcinomas were present in the MSA controls (Fig. 4*a*). At this time, the IL-12 regimens resulted in distinct pathological features. Either *in situ* carcinomas or invasive carcinomas were evident in the mammary glands of mice from the chronic and late treatment groups (Fig. 4,*b* and *c*). These lesions were smaller and less widely distributed than those in MSA controls and were even less pronounced in the chronic treatment group. In contrast, a restrained atypical hyperplasia with foci of carcinoma *in situ* only was evident in mice from the early treatment group (Fig. 4*d*).

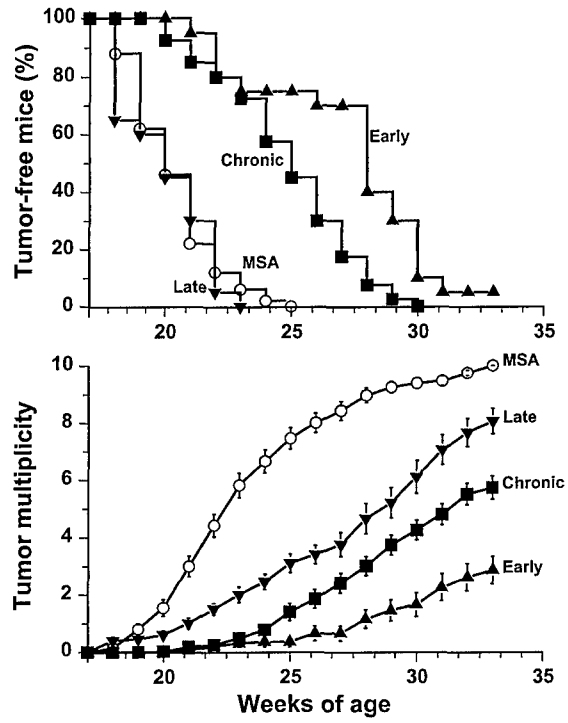

Fig. 3. Progression of mammary carcinogenesis in BALB-neuT mice receiving chronic, late, or early administration of IL-12. The percentage of tumor-free mice (*top panel*) and the mean number of palpable mammary carcinomas/mouse (calculated as the cumulative number of incident tumors/total number of mice; *bottom panel*) are shown. Fifty mice were MSA controls. There were 40 mice in the chronic treatment group, and 20 mice in both the early and the late treatment groups. Statistical analysis in the *top panel* shows that both early and chronic curves are significantly different (at least $P < 0.0005$ Mantel-Haenszel test) from the MSA curve, whereas the late curve is not significantly different. After week 21, all values in the *bottom panel* of early, chronic, and late treatment groups are significantly different from the corresponding values of the MSA group at least, ($P < 0.05$ Student's t test).

Inhibition of Tumor Vasculature in BALB-neuT Mice. This IL-12-induced delay of carcinogenesis closely fits the inhibition of tumor angiogenesis as assessed by direct microvessel count (Table 1). At 15 weeks, mammary glands from the MSA controls displayed vigorous capillary sprouts inside the atypical hyperplastic areas, whereas only a few capillaries surrounded the foci of *in situ* carcinoma. Minor vascular damage and inhibition of angiogenesis were evident in mice from the late treatment group. In contrast, a defective vascular network and a moderate reduction and a marked reduction of the number of microvessels were evident in mice from the chronic and early treatment groups (Fig. 2,*a–c*). These differences diminished markedly at the 25th week, when evident tumors were present in all treatment groups (Table 1; Fig. 2, *d–f*).

Proliferative Rate of BALB-neuT Tumors. To evaluate whether IL-12 treatments affect the growth rate of evident tumors, the time required by a tumor with a mean diameter of 4 mm to reach 8 mm in mean diameter was calculated for the first tumor in each mouse. IL-12 increased tumor doubling time, but this increase was too small to be significant. PCNA immunostaining to assess the rate of epithelial cell proliferation was mainly detected in the peripheral cell layer of neoplastic lobules in untreated mice and in all treatment groups. Evaluation of PCNA-positive cells, also failed to disclose appreciable differences among the treatments (Table 1).

Efficacy of Lower IL-12 Doses in BALB-neuT Mice. Because IL-12 appears to effectively inhibit the progression of HER2/*neu* carcinogenesis, the dose range in which such an inhibition is achieved was evaluated. When early treatment was performed using 10 and 50 times lower doses of IL-12, no delay in the appearance of the first tumor or reduction of the number of mammary glands with a palpable tumor was found, but a slight delay in tumor onset was seen (Fig. 5).

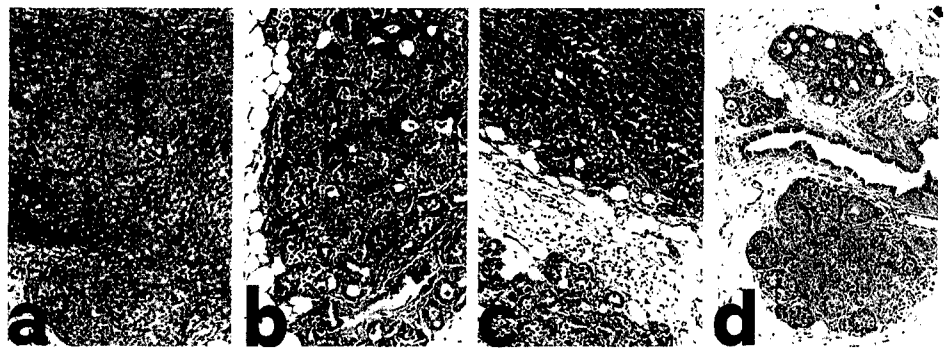

Fig. 4. Histopathology of mammary lesions in 25-week-old BALB-neuT mice. Invasive carcinomas formed by a uniform population of round cells grouped in alveolar structures are evident in the mammary glands of MSA controls (*a*). Multiple foci of carcinoma *in situ* associated with some hyperplastic islets were the main feature in mice from the chronic treatment group (*b*), whereas both invasive carcinomas and large carcinoma *in situ* were present in mice from the late treatment group (*c*). A restrained hyperplasia and a few foci of carcinoma *in situ* are evident in mice from the early treatment group (*d*).

Table 1 *Microvessel counts, expression of PCNA, and tumor doubling time in mammary tumors of BALB-neuT mice treated with IL-12*

	IL-12 treatment			
	MSA only	Chronic treatment	Late treatment	Early treatment
Microvessel count[a]				
15th week	22 ± 3	13 ± 2[b]	19 ± 3	9 ± 2[b]
25th week	13 ± 2	12 ± 2	13 ± 4	11 ± 2
% of PCNA immunoreactivity				
30th week[c]	23 ± 6	21 ± 5	27 ± 9	22 ± 4
Doubling time of the diameter (4–8 mm) of the first mammary tumor	8 ± 5	12 ± 8	25 ± 19	25 ± 18

[a] Performed on cryostat sections with antiendothelial (CD31) monoclonal antibody. At least 10 fields/sample were counted. Values are expressed as mean ± SD of five 15- and 25-week-old mice.

[b] Values are significantly different ($P > 0.001$) from those of MSA controls.

[c] Performed on paraffin-embedded tissue sections with anti-PCNA monoclonal antibody.

Prevention of Carcinogenesis in FVB-neuN Mice. In FVB-neuN mice, the overexpressed *neu* proto-oncogene induces mammary carcinomas with a much longer latency time. Until the 22nd week, the mammary glands of these mice are histologically normal, whereas foci of atypical hyperplasia and carcinoma *in situ* become evident in a few glands of 25-week-old mice. Randomly, a few of them progress slowly toward invasive carcinoma, and a mean number of 2.5 tumors/mouse is evident at the 60th week. The 6-week-old and 22-week-old IL-12 treatments began when FVB-neuN mice were still free from macroscopic or microscopic mammary lesions (17). Both treatments significantly reduced tumor incidence and multiplicity as compared with MSA controls (Fig. 6). In contrast, 28-week-old treatment was almost ineffective. It began when focal hyperplasia and carcinoma *in situ* were already a common finding.

DISCUSSION

With distinct kinetics, transgenic female mice carrying the activated (BALB-neuT) or the proto-oncogene (FVB-neuN) rat HER2/*neu* under the MMTV promoter progress toward a consistent pattern of spontaneous mammary carcinogenesis that recapitulates a few features of the development of human mammary carcinoma (16). In both types of mice, IL-12 delays the onset and counteracts the multiplicity of mammary carcinomas. The present findings extend and confirm previous observations of mice treated with IL-12 during the whole progression of mammary carcinogenesis (16). Noguchi *et al.* (15) have shown previously that a similar IL-12 treatment also inhibits chemical carcinogenesis in mice.

Because these findings suggest that administration of IL-12 is of significance in hampering the progression of preneoplastic lesions, the specific issue addressed here was to define the stage of tumor progression in which these mechanisms are most effective. Should IL-12 administration be proposed as a preventive measure in healthy individuals with genetic risk of cancer patients, or can it also be of benefit once overt preneoplastic lesions are diagnosed? This is a significant question because genetic screening programs are singling out healthy individuals with genetic risk of cancer (18), and early diagnosis programs are detecting preneoplastic lesions (19).

As a result of the activated *neu* transgene, BALB-neuT mice display mammary cell atypia virtually from birth. The efficacy of IL-12 treatments in these mice suggests that the evolution of the tumor:host angiogenic relationship, rather than the intrinsic proliferative properties of transformed mammary cells, is the point of no return for IL-12 activity. In effect, the present findings suggest that at least part of this activity is due to the ability of IL-12 to inhibit the angiogenesis associated with mammary hyperplasia.

Around the 2nd week, almost all mammary glands of BALB-neuT mice display multiple foci of ductular atypical hyperplasia. Between

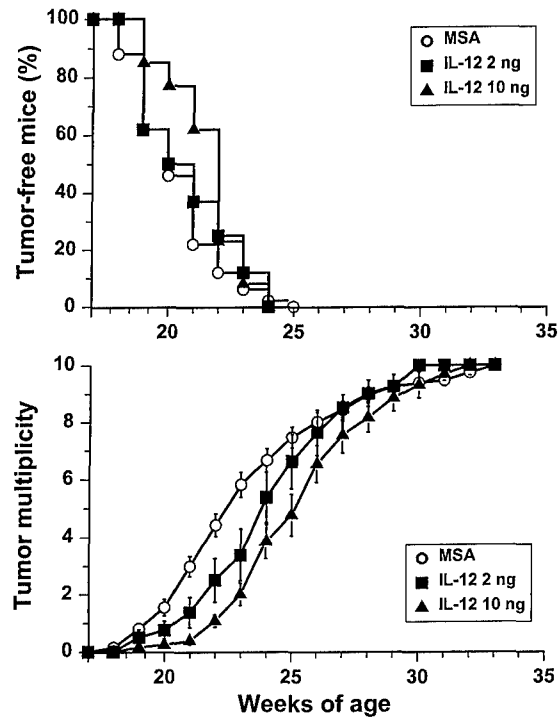

Fig. 5. Progression of mammary carcinogenesis in BALB-neuT mice from the early treatment group that received daily i.p. injections of 10 and 2 ng of IL-12. The percentage of tumor-free mice (*top panel*) and the mean number of palpable mammary carcinomas/mouse calculated as the cumulative number of incident tumors/total number of mice; (*bottom panel*) are shown. Each group consists of 10 mice. In the *top panel*, both IL-12 curves were not significantly different from the MSA curve by the Mantel-Haenszel test. Values of the groups receiving 10 and 2 ng of IL-12 are significantly different ($P > 0.001$) from corresponding values in mice of the same treatment group receiving 50 (first course) and 100 (following courses) ng of IL-12 (Fig. 2).

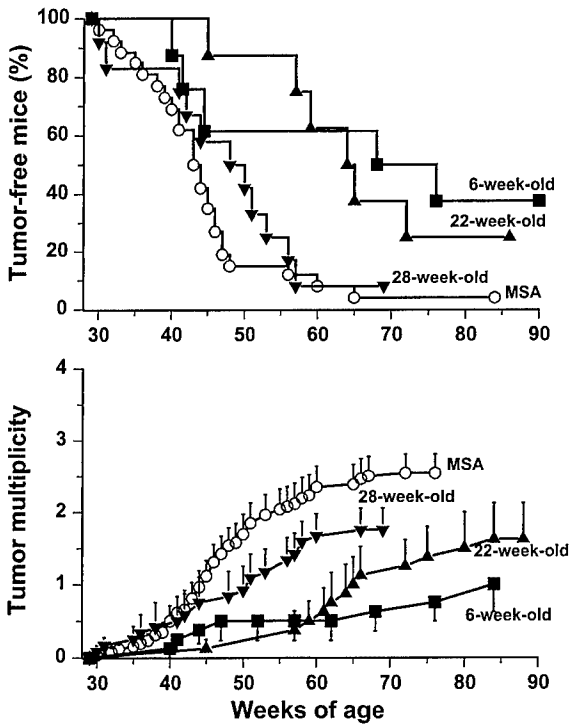

Fig. 6. Progression of mammary carcinogenesis in FVB-neuN mice receiving IL-12 treatment starting at 6 (6-week-old), 22 (22-week-old), or 28 (28-week-old) weeks of age. The percentage of tumor-free mice (*top panel*) and the mean number of palpable mammary carcinomas/mouse calculated as the cumulative number of incident tumors/total number of mice (*bottom panel*) are shown. Twenty-six mice were in the MSA control group, 12 mice were in the 28-week-old group, and 8 mice were in both the 6-week-old and the 22-week-old groups. In the *top panel*, both 6-week-old and 22-week-old curves were significantly different at least, ($P < 0.025$ Mantel-Haenszel test) from the MSA curve, whereas the 28-week-old curve was not statistically different from the MSA curve. In the *bottom panel*, after week 48, all values of both 6-week-old and 22-week-old groups were significantly different ($P < 0.05$, Student's t test) from the corresponding values of the MSA treatment group. No value of the 28-week-old curve was significantly different from the corresponding value of the MSA treatment group.

the 13th and 17th weeks, hyperplasia progresses to *in situ* carcinoma (Ref. 16; present study). Immunohistochemical staining with anti-CD31 monoclonal antibody shows that rich microvascularization inside preneoplastic lesions corresponds with their progression toward carcinoma, as shown in other tumor systems (23). This progression phase appears to be particularly appropriate for an angiostatic intervention (24, 25). Indeed, the most significant delay in tumor onset and progression is observed with the early treatment, when IL-12 courses given from the 2nd to the 14th week induced both a scanty vascularization and poorly developed hyperplastic foci.

The importance of the timing of IL-12 administration was further assessed with FVB-neuN mice, in whom an overexpressed *neu* proto-oncogene induces mammary carcinomas after a markedly longer latency. The 6-week-old treatment consists of a lifetime administration of IL-12 and is conceptually similar to the chronic treatment of BALB-neuT mice. Although the first course was markedly delayed on the 22-week-old treatment, it still started before an evident spreading of preneoplastic lesions. Both treatment schedules delay the onset of carcinomas and their multiplication. The period between the 22nd and the 28th week appears to be of critical importance because the 28-week-old protocol confers only a negligible protection. During these 6 weeks, in fact, normal mammary glands progress toward atypical hyperplasia and then toward carcinoma *in situ* and invasive carcinoma. Palpable tumors are first detected at 30 weeks.

The equivalent results from BALB-neuT and FVB-neuN mice suggest that IL-12 effectively inhibits mammary carcinogenesis when its administration accompanies the angiogenic switch. Its antiangiogenic effect appears to rest on the increased serum levels of IFN-γ and tumor necrosis factor-α released by activated T lymphocytes and natural killer cells (5, 7). The antiangiogenic (4, 8) and angiotoxic (26) activity of these two cytokines is stronger on those fragile capillary sprouts, which go with the shift from the preneoplastic to the neoplastic condition. Downstream mediators elicited by IL-12 may also act on neoplastic cells, in which they down-regulate the production of proangiogenic molecules (7, 27) and up-regulate the release of antiangiogenic factors such as IFN-inducible protein 10 and monokine induced by IFN-γ (7, 14). After the transition from hyperplasia to *in situ* and invasive carcinoma, capillary sprouting becomes restrained. The poor efficacy of late treatment in both BALB-neuT and FVB-neuN mice may depend on the lower sensitivity of mature and differentiated blood vessels of the more advanced neoplastic lesions to IL-12-induced angiostasis.

The decreased number of microvessels per microscopic field in both *in situ* and invasive carcinoma in comparison to hyperplastic areas suggests that this type of carcinoma, once developed, no longer requires a profuse vascular supply. The few vessels of the stroma of neoplastic lobular-alveolar structures are enough to sustain their relatively low rate of proliferation. In contrast, blood supply is a critical factor for most fast-growing transplantable tumors, even during their later stages. This necessity may account for the high efficacy of IL-12 against these tumors, even when they are large (3, 7). With tumors that progress slowly, antiangiogenic activity is only efficacious in specific progression stages (24). This narrow window of activity might account for the ineffectiveness of IL-12 in the management of human cancer, because only patients bearing advanced tumors are enrolled in clinical trials (28).

The antitumor action of IL-12 is not confined to its indirect influence on endothelial cells. Directly or through secondary cytokines, its triggers lytic activity and mediator release in a variety of tumor-infiltrating leukocytes, thus offsetting the continuous generation of new transformed cells (7, 10–12). The efficacy of the hampering of tumor progression by IL-12 probably rests on the sum of its activities and not simply on the blocking of tumor neoangiogenensis, as important as this may be. In effect, further subdivision of the early protocol in shorter treatment periods markedly reduced IL-12 efficacy (data not shown).

The lower efficacy of chronic *versus* early treatment could indicate that continuous IL-12 administration is suppressive (29), although this possibility is not endorsed by the results in FVB-neuN mice. It should be noted that from the second course, BALB-neuT and FVB-neuN mice received 100 ng/day IL-12 (*i.e.*, around 4.5–7.7 μg/kg). This dose is well tolerated, and almost no side effects were manifested (7, 16). It is probably close to the optimal active dose, because a 10- or 20-fold reduction abolishes its activity.

In conclusion, our data suggest that IL-12 effectively impairs the *neu* oncogene-driven progression of mammary carcinogenesis by interfering with the passage from atypical hyperplasia to invasive carcinoma. This interference appears to depend largely on indirect inhibition of tumor-associated angiogenesis. Its diminished efficacy in more advanced lesions and the dose range required pose some constraints on the use of IL-12 as an immunological alternative to current management of already manifest neoplastic lesions. Nevertheless, the efficacy of IL-12 points to enhancement of nonspecific immunity as an effective way to prevent mammary tumors in individuals at risk. Lifetime administration is not required for genetically determined cancers with a long natural history; instead, a precise definition of the carcinogenic events may allow preventive treatments starting relatively late in the life of individuals at risk.

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