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in Transgenic Mice

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13. ABSTRACT (Maximum 200 words) Our purpose is to explore the possibility of employing immunological intervention to hamper the carcinogenic process and the growth of spontaneous mammary carcinomas in mice. The final scope is to get enough information to decide whether a similar approach may be applied in humans at risk. The mammary glands of female mice transgenic for the rat HER2/neu oncogene progress to carcinomas. Whether, at what stage and how nonspecific immunity elicited by IL-12 hampers this progression was evaluated in Balb/c and FVB Mice. In both mouse strains, 5-day courses of 50/100 ng IL-12/day inhibited carcinogenesis when they coincided with the progression of early lesions. Inhibition appears to mostly depend on IL-12's ability to interfere with early tumor angiogenesis. Later treatments are less effective and lower IL-12 doses are useless. The efficacy of specific immunity has been evaluated by immunizing these mice with peptides from rat Her-2/neu product admixed with cytokines able to recruit dendritic cells. Adenocarcinoma cells expressing the rat Her-2/neu and secreting various cytokines are currently engineered. In the meantime, immunization of normal and transgenic mice with allogeneic rat Her-2/neu tumor cells inhibits a subsequent challenge with syngeneic Her-2/neu adenocarcinomas. As specific immunity can be elicited, its potential against spontaneous carcinomas is being evaluated.			
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FOREWORD

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Guido Foduri

July 27, 1999.

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4 TABLE OF CONTENTS

1.	Front Cover.....	1
2.	Standard Form (SF) 298, Report Documentation Page.....	2
3.	Foreword.....	3
4.	Table of Contents.....	4
5.	Introduction.....	5
6.	Body Task 1, Prevention of mammary carcinoma with recombinant IL-12.....	5
	Task 2-3, Prophylactic vaccination with cytokine gene-transduced tumor cells....	13
	Task 4, Vaccination with <i>neu</i> peptides.....	15
7.	Key research accomplishments.....	17
8.	Reportable outcomes.....	18
9.	Conclusions.....	19
10.	References.....	20
11.	Appendices.....	21
	• Appendix #1. Boggio et al., Interleukin 12-mediated prevention of spontaneous mammary carcinomas in two lines of Her-2/ <i>neu</i> transgenic mice. J. Exp. Med. 188:589, 1998.....	21
	• Appendix #2. Di Carlo et al., Analysis of mammary carcinoma onset and progression in Her-2/ <i>neu</i> oncogene transgenic mice Lab. Inv., 1999 in press.....	26
	• Appendix #3. Lollini and Forni, Specific and nonspecific immunity in the prevention of spontaneous tumors. Immunol. Today, 1999 in press.....	37
	• Appendix #4. Boggio et al., Ability of systemic IL-12 to hamper progressive stages of mammary carcinogenesis in Her2/ <i>neu</i> transgenic mice, (to be submitted).....	41
	• Appendix #5. Nanni et al., p185 ^{neu} protein is required for tumor and anchorage-independent growth, not for cell proliferation of transgenic mammary carcinoma, (to be submitted).....	49

5 INTRODUCTION

Current studies are leading to the identification of gene mutations that predispose to cancer and hence are holding out the possibility of identifying not-yet patients with a defined genetic risk. This possibility is a reality in breast cancer, one of the most common malignancies in women. In the absence of effective preventive options, this probing of the human genome is raising dramatic issues (1). On the other hand, identification of the mutated gene and its altered or amplified products is also providing a new opportunity to perform immunological interventions against oncogene products in an unprecedented setting. The purpose of this program is to evaluate the potential of immunoprevention of tumors in individuals with a specific risk of cancer. The concept of "immunoprevention" of tumors is innovative. It has come forward as a rational development of previous work with transplantable tumors. Vaccination with cytokine-engineered tumor cells confers a strong protection against a subsequent challenge with parental adenocarcinoma cells (2, 3). Immunization of tumor-free hosts is highly successful. The treatment of tumor-bearing hosts, however, loses efficacy as the tumor progresses (4). We propose to exploit the protection elicited by immunologically treatments in an "immunological correct" context, i.e. before tumor onset (5). In effect, previous data suggest that the appropriate time to intervene with immune prevention is when a genetic predisposition to a specific cancer is detected and when preneoplastic alterations are found in the mammary gland. The females of two strains of mice (Balb/c, H-2^d; FVB, H-2^q) transgenic for the activated (Balb-NeuT) and the amplified (FVB-NeuN) rat *Her-2/neu* oncogene display neoplastic mammary gland alterations that progress through atypical hyperplasia to in situ lobular carcinoma and invasive carcinomas (6) (Appendix #1). Many aspects of this progression closely mimic the progression of human mammary carcinomas. Characterization of these two models of *Her-2/neu* mammary carcinogenesis (7) (Appendix #2) allows one to assess the potential of specific and non-specific immunological interventions started at different stages of carcinogenesis progression. Our final scope is to get enough information from these preclinical models of spontaneous carcinogenesis to decide whether a similar tumor prevention approach may be applied in humans at risk. This issue has been discussed in depth in the light of the initial data obtained in the first year of this research program, see ref. 5 (Appendix #3).

6 BODY

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

The specific aim is to prevent mammary carcinoma in Balb-NeuT and FVB-NeuN female mice by repeated systemic administrations of IL-12. During the period in which this grant application was processed, the preliminary data quoted in the program have been confirmed and published (6) (Appendix #1). The initial part of the study was not financed by this Army grant. Even so, it is here reported as Appendix #1 because it contains basic information that supplements and clarifies the features of Task 1. We observed that treatment of Balb-NeuT females with IL-12 (5 daily i.p. injections, 1 week on, 3 week 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 on the 21st in FVB-NeuN mice markedly delayed tumor onset and reduced tumor multiplicity. Analogous results were obtained in immunocompetent and CD8⁺ T lymphocyte-depleted mice. In both transgenic lines, tumor inhibition was associated with mammary infiltration of reactive cells, production of cytokines and iNOS, and reduction in vessel number together with a massive hemorrhagic necrosis. These findings opened a few key questions:

- What are the pathological features of the lesion displayed by Balb-NeuT and FVB-NeuN mice ?
- What is the potential of IL-12 during carcinogenesis progression?
- What are the immune mechanisms responsible for the inhibition of carcinogenesis provoked by IL-12 ?

To answer these questions, the steps and the features of the Her-2/*neu* mammary carcinogenesis in Balb-NeuT and FVB-NeuN mice were first defined (7). Technical and specific features are reported in (Appendix #2). The suggestion emerging from this study is that despite the difference in tumor kinetics, histological and ultrastructural examination of mammary neoplasias arising in Balb-NeuT and FVB-NeuN mice showed that they give raise to morphologically indistinguishable lobular carcinomas. Diagnosis of lobular carcinoma is based on replacement by neoplastic cells of the normal epithelium of acini and intralobular ductules. The acinar outlines remain distinct and separate from one another with persistence of intervening delicate stroma. Widespread atypical hyperplasia of small lobular ducts and lobules of the mammary gland of Balb-NeuT mice is already evident at 3 weeks and is characterized by proliferation of a relatively uniform and stratified population of round epithelial cells with a solid growth pattern and no formation of epithelial bridges. Starting at the 11th week, the ductules and acini within the lobules are distended and occluded by the proliferation of a loosely cohesive, uniform cell population. Ductular and acinar outlines remain distinct and separate from one

another, with persistence of intervening stroma. These features are distinctive of lobular carcinoma "in situ". At nearly the 20th week, alveolar grouping of neoplastic cells, lacking the myoepithelial cell lining, infiltrates the surrounding adipose tissue.

FVB-NeuN mice display normal ductular and lobular structures until the 35-37 week, when epithelial hyperplasia appears and progresses first to lobular carcinoma in situ and then to invasive lobular alveolar carcinoma. The histological features of this carcinoma are similar to those observed in Balb-NeuT mice, though the proliferating cell population displays minor variations in size and cytoplasm staining.

Balb-NeuT carcinomas strongly express the cell-cell adhesion molecule E-cadherin, whereas it is absent in FVB-NeuN carcinomas. This feature could explain the observation that the overexpressed rather than the activated *Her-2/neu* gene confers an enhanced metastatic potential on mammary tumor cells. In both strains, the genetic alteration leads to proliferation of the epithelial cells located in the lobular structures, similar to type 2 lobules, which contain almost all cells expressing *Her-2/neu* product. *Her-2/neu* triggered epithelial cell proliferation is evidenced by a widely distributed expression of PCNA in the more differentiated lobular structures. A tight connection between this cell proliferation and activation of angiogenesis is evident. Angiogenesis appears begin in hyperplastic foci before overt tumor formation. Several microvessels expressed the $\beta 3$ subunit of $\alpha v\beta 3$ integrin, which promotes endothelial cell migration and angiogenesis, and protects from apoptosis. Neovascularization is probably activated by bFGF and VEGF produced by hyperplastic epithelial cells, whose expression is confined to the basal neoplastic epithelial cell layer close to the intervening stroma. During carcinogenic progression, the extracellular matrix could sequester bFGF and impede its angiogenic activity. The mean number of microvessels/microscopic field was appreciably reduced in lobular carcinoma compared with the preceding hyperplasia, in which the extracellular matrix laminin and collagen type IV are less represented (7) (Appendix 2).

Diagnosis of lobular mammary carcinoma with a progression analogous to that in women and characterized by a peculiar angiogenesis pattern could help the design of immunological strategies to inhibit carcinogenesis at individual stages. We therefore set out to define the progression stage in which these 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 (1) and early diagnosis programs are detecting

pre-neoplastic lesions (8). Technical and specific features related to this task of the program are reported in detail in ref. 5 and Appendix #3 and in Appendix #4.

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.

Until the 35-37 week the mammary glands of FVN-NeuN mice are normal. A random slowly progress to invasive carcinoma, and a mean 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 lesion-free. Both treatments significantly reduced tumor incidence and multiplicity as compared to MSA controls. By contrast, the "28-week-old" treatment was almost ineffective. It began when focal hyperplasia and carcinoma in situ are already a common finding.

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. Indeed, the most significant delay in tumor onset and

progression is observed with the "Early" treatment, which induced both a scanty vascularization and poorly developed hyperplastic foci.

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 downregulate the production of pro-angiogenic molecules and upregulate the release of anti-angiogenic factors, such as IP-10 and MIG (2). 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 (9).

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 (2, 10). The efficacy of IL-12 probably rests on the sum of its activities, and not simply on blocking of tumor neoangiogenesis, 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 (6, 10). It is probably close to the optimal active dose, since a ten or twenty-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 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 preventive treatments to be started relatively late in the life of individuals at risk.

The next question now being investigated is definition of the immune mechanisms responsible for inhibition of carcinogenesis by IL-12. Two approaches are being followed:

- Selective and persistent immunosuppression
- Production of gene KO mice expressing the rat *Her-2/neu* oncogene.

To study the influence of persistent and selective immunosuppression on the development of mammary lobular carcinomas, Balb-NeuT males were crossed with B6 mice. Despite the acquisition of a new H-2^b major histocompatibility complex (11), these F1 hybrid females (B6B-

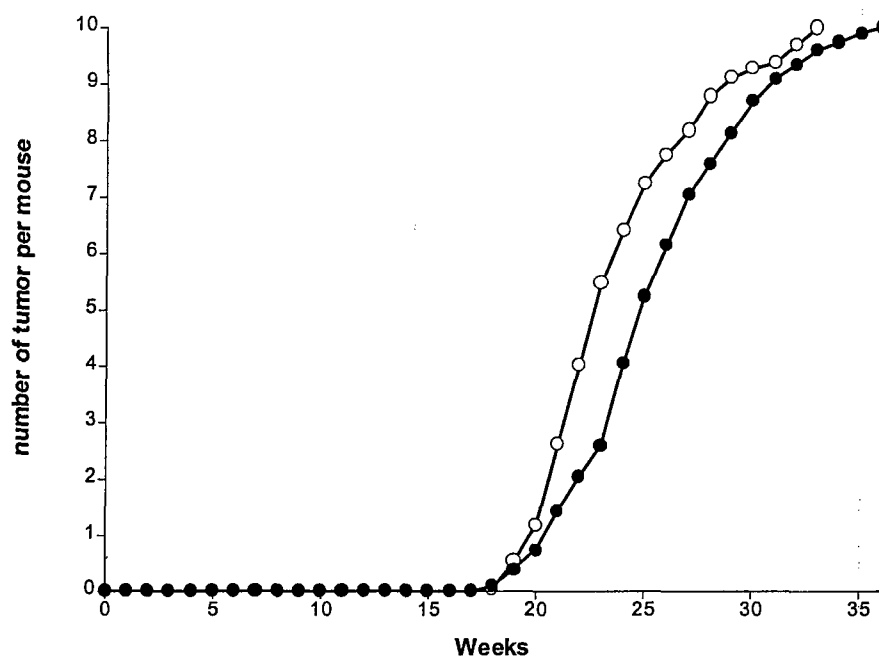


Fig. 1. Temporal kinetics of the development of mammary lobular carcinomas in B6B-NeuT mice (black circles) and Balb-NeuT (white circles). Each group: >20 mice

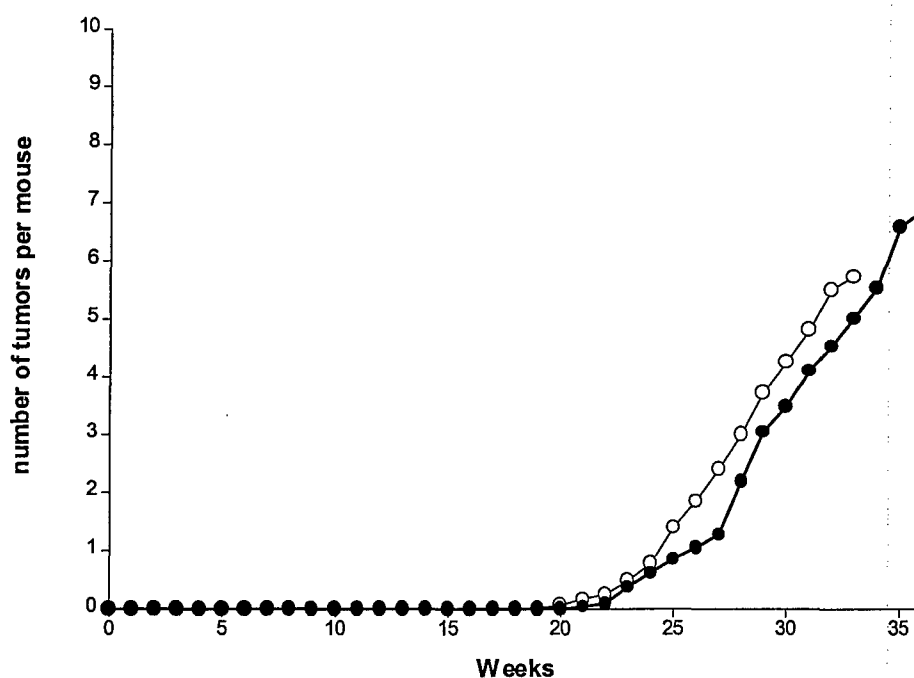


Fig. 2. Effect of chronic IL-12 administration (5 daily i.p. injections, 1 week on, 3 week off; the first course with 50 ng IL-12/day, the following with 100 ng IL-12/day, seven courses total begun at the 2nd week of age) on the development of mammary carcinomas in B6B-NeuT (black circles) and Balb-NeuT (white circles) mice. Each group: >25 mice

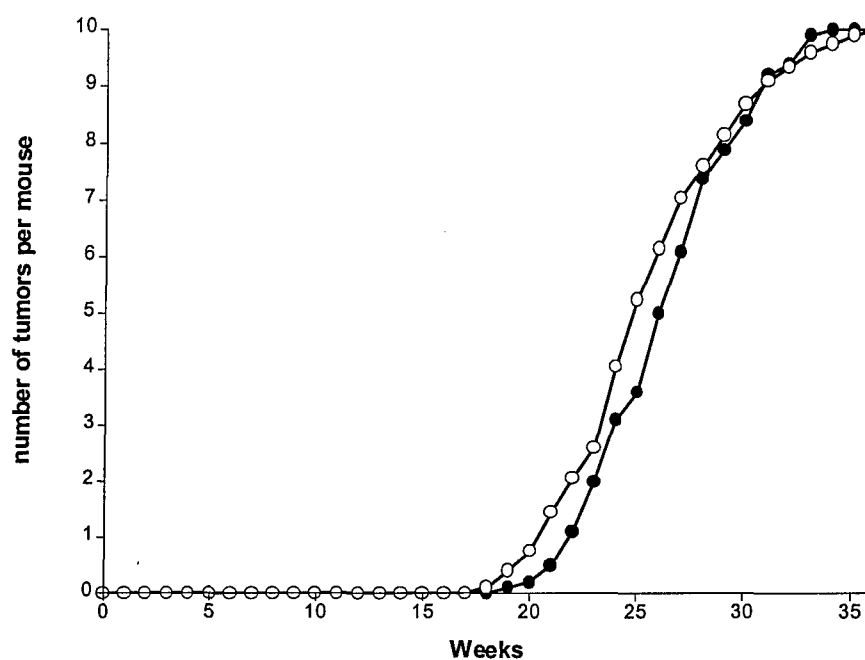


Fig.3. Effect of chronic α NK 1.1 administration on the development of mammary carcinomas in B6B-NeuT mice (black circles) compared with the controls B6B-NeuT mice (white circles). Each group: 17 mice

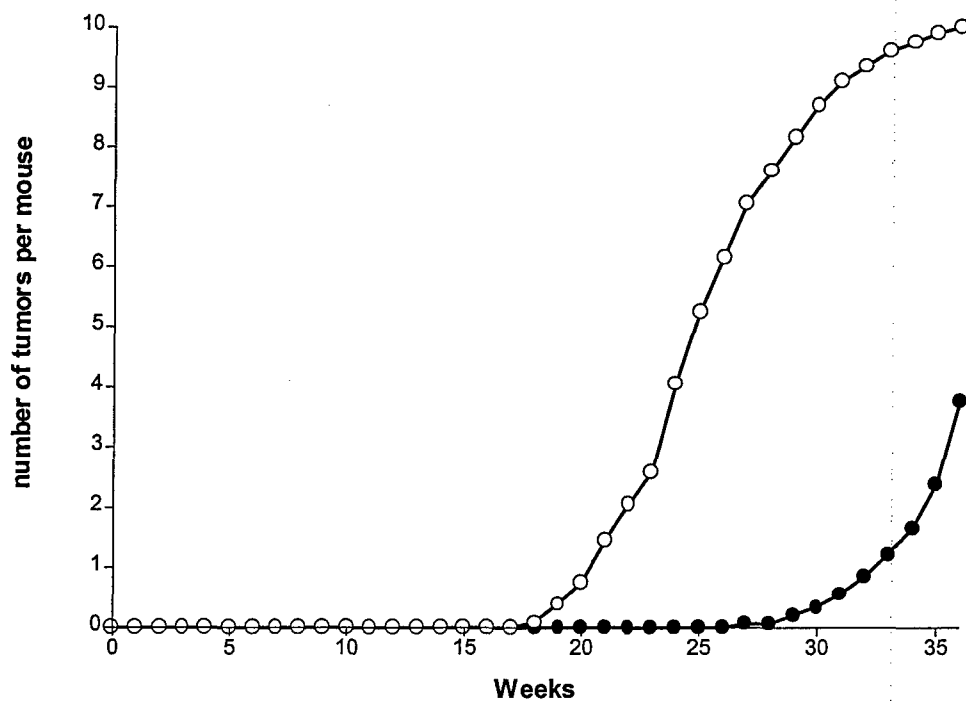


Fig.4. Neonatal thymectomy and anti CD4 and anti CD8 treatment enhanced the protective ability of IL-12 in B6B mice (black circles) compared with the controls B6B-NeuT mice (white circles). Each group: 15 mice.

observed in Balb-NeuT mice (Fig. 2). B6B-NeuT mice are being used to examine the effect of repeated treatment with mouse anti NK 1.1 monoclonal antibody to inhibit NK cell activity. Unexpectedly (2, 6), depletion of NK.1.1 cells did not affect the ability of IL-12 to impair tumor development (Fig 3). Even more surprising was the finding that neonatal thymectomy and anti CD4 and anti CD8 treatment enhanced the protective ability of IL-12 (Fig.4). The immunological and immuno-endocrinal reasons for this paradox are currently studied.

The role of the immunological components in inhibition of carcinogenesis by IL-12 is also being investigated by breeding Balb-NeuT mice bearing KO genes. The following mice will be available in a few months:

- Balb-NeuT IFN- γ KO [in collaboration with Dr. Mario P. Colombo, Milan, Italy (12)]
- Balb-NeuT TNF- α KO [in collaboration with Dr. Francis Balkwill, London, UK (13)]
- Balb-NeuT MCP-1 KO [in collaboration with Dr. Bernard Rollins, Boston, MA (14)]
- Balb-NeuT CD1 KO [in collaboration with Dr. Luc Van Kaer, Nashville, TN (15)]

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

The aim is to:

- a) induce in healthy Balb-NeuT (Task 2) and FVB-NeuN (Task 3) female mice a specific immunity against rat Her-2/*neu* gene product
- b) determine whether this immunity inhibits spontaneous carcinogenesis and cures initial neoplastic lesions.

Anti- rat Her-2/*neu* vaccines have been sought in two ways:

a) We have transduced the Balb/c mammary adenocarcinoma TSA-pc with many genes coding for cytokines and membrane antigens (2-4, 10, 16). The original plan was to transduce rat Her-2/*neu* oncogene in these TSA clones and test their efficacy in vivo. p185^{neu} is the protein product of the Her-2/*neu* oncogene. RT-PCR of TSA-pc showed that they express high levels of mouse p185^{neu} mRNA. When we transduced TSA cells with the rat Her-2/*neu* gene, rat p185^{neu} was always poorly expressed. The use of TSA clones transfected with various cytokines has therefore been temporarily suspended.

b) Clones from spontaneous mammary carcinomas growing in Balb-NeuT and FVB-NeuN mice will be obtained and transduced with genes coding for various cytokines and membrane antigens (16, 17). A few cell lines from adenocarcinomas of FVB-NeuN mice were

established and cloned. Two of these clones were particularly interesting: N202.1A and N202.1E. N202.1A cells express on their membrane large amounts of p185^{neu}, N202.1E cells express none. The growth characteristics of these two clones and the technical and specific features related to this task of the program are reported in detail in Appendix #5. A few cloned lines have been established from Balb-NeuT mice. One line (TUBO cells), expressing high levels of rat p185^{neu} has been mainly studied. TUBO cells injected subcutaneously in the inguinal region of Balb/c mice give rise to progressively growing solid tumors (Fig. 5) with the characteristics of spontaneous infiltrating lobular carcinoma (7). A similar growth pattern was observed in Balb-NeuT mice (Fig.5).

Creation of p185^{neu} positive and negative clones has enabled the investigation of specific immunity to be speeded up through the use of transplantable tumors prior to planning studies of the inhibition of spontaneous tumors. Immunization of Balb/c mice with 1×10^6 N202.1A, but not with N202.1E cells, confers a specific protection against a subsequent challenge with TUBO cells (Table 1). This experiment is now being repeated in Balb-NeuT mice, which are fully tolerant to rat Her-2/*neu* products. Since N202.1A cells are a good immunogen in allogeneic mice, we are seeking to enhance their immunogenicity by engineering them with the genes of

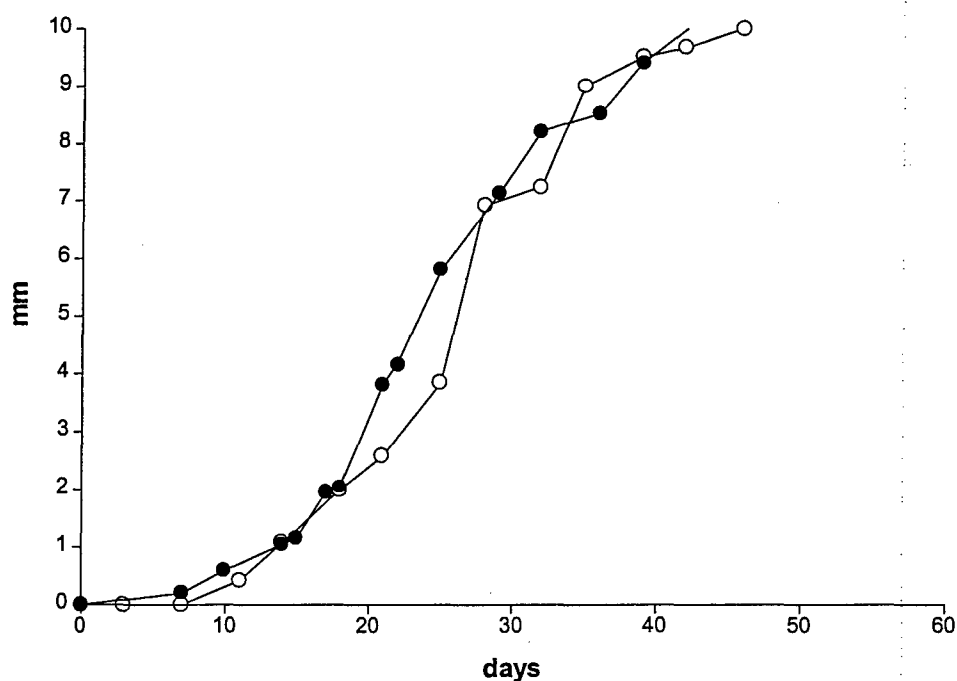


Fig. 5. Growth pattern of 1×10^5 TUBO cells in Balb/c (black circles) and Balb-NeuT mice (white circles). Each group: 10 mice

Table.1. Subcutaneous growth of TUBO cells in Balb/c mice immunized with N202 cells from FVB mice. Mice were immunized 15 days before the challenge with 1×10^6 cells and challenged with 1×10^5 TUBO and TSA cells.

Subcutaneous vaccination with: -----	Tumor free mice after a challenge with:	
	TUBO cells -----	TSA-cells -----
None	0/5	0/5
N202.1A	5/5	0/5
N202.1E	0/5	0/5

several cytokines. N202.1A cells engineered to release IL-2, IFN- γ and IL-15 are now available. We are expecting to obtain N202.1A cells releasing LEC, a novel chemokine (18). All these clones have been transfected using both Lipofectin reagent (Life Technologies, North Andover, MA) and Calcium Phosphate transfection system (Life Technologies) with pCDNA3 vectors (Invitrogen, San Diego, CA) in which cytokine genes have been inserted. We have also tried to transfect TUBO cells, but they appear to be refractory to this method of transfection. We are now looking to see whether direct transduction with a Helium Gene Gun yields better results.

TASK 4. VACCINATION WITH HER-2/NEU PEPTIDES

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^a has not been thoroughly studied) we have studied this approach in Balb-NeuT mice, of H-2^d background. 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 (19). 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).

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 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 1×10^7 syngeneic spleen cells (Spc) pulsed overnight with peptides. One week after the last immunization, a few mice were sacrificed to assess Spc cytotoxicity against TUBO cells after six days in vitro restimulation with mitomycin-C treated (Sigma, St. Louis, MO; 100 µg/10⁷ cells/ml for 30 min) TUBO cells. Cytotoxic activity was expressed as Lytic Units₂₀ (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 2).

Table 2. Cytotoxicity against TUBO cells of Spc from vaccinated mice after in vitro stimulation with Mitomycin-C treated TUBO cells.

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

Despite this feeble reactivity, Spc from immunized mice do not inhibit the in vivo growth of TUBO cells (Table 3).

Table 3. 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 $\pm$ 1	48 $\pm$ 8
Spc not pulsed	5/5	24 $\pm$ 2	49 $\pm$ 2
Spc pulsed with P114-002	5/5	26 $\pm$ 2	54 $\pm$ 7
Spc pulsed with P114-003	5/5	25 $\pm$ 1	51 $\pm$ 3
Spc pulsed with P114-004	5/5	26 $\pm$ 1	48 $\pm$ 9

^aLatency time: time in days between the challenge and the appearance of tumors > 3 mm mean diameter. ^bSurvival 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.6, 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.

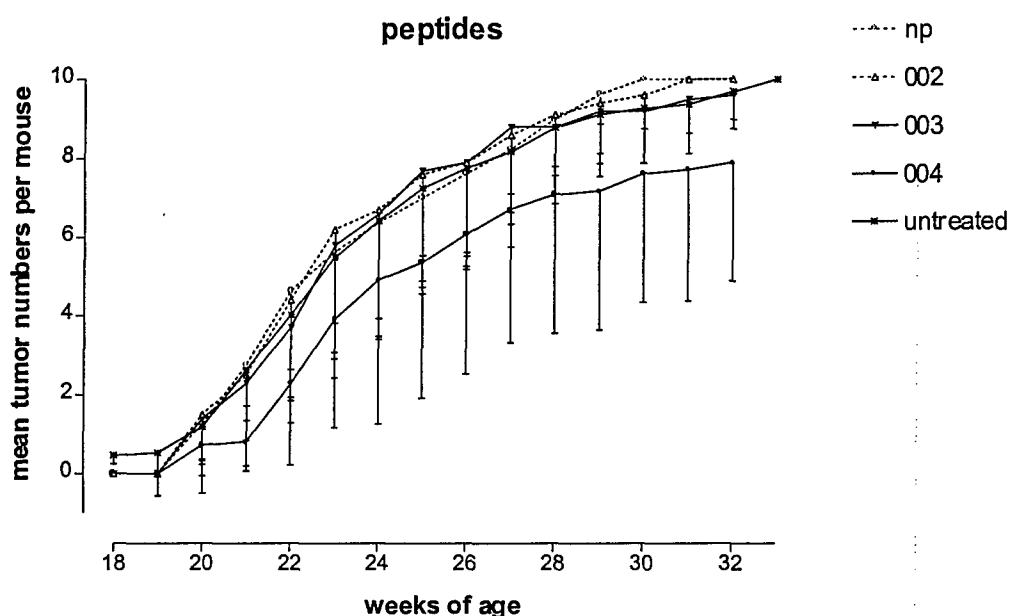


Fig . 6. Effect of vaccination with Spc not pulsed (np) or pulsed with the various peptides (002, 003, 004) on the development of spontaneous mammary carcinomas in Balb-NeuT mice. Each group: 10 mice

7 KEY RESEARCH ACCOMPLISHMENTS

- Characterization of the progressive steps of Her-2/*neu* carcinogenesis and analogies with the progression of lobular carcinomas in women
- An effective inhibition of carcinogenesis rests on non-specific immunity elicited by IL-12
- Importance of the time of IL-12 administration
- IL-12 treatments during progression of early preneoplastic lesions are very effective while later treatments are much less so
- Inhibition appears to mostly depend on IL-12's ability to interfere with early tumor angiogenesis
- Daily doses of 100 ng IL-12 /mouse are effective, doses of 10 and 2 ng are useless.

- Establishment and characterization of cloned p185^{neu} positive (N202.1A) and negative (N202.1E) tumor cells from FVB-NeuN spontaneous mammary carcinomas
- Production of N202.1A p185^{neu} positive cells engineered to release IL-2, IFN- γ , IL-15
- Establishment and characterization of cloned p185^{neu} positive tumor cells from Balb-NeuT spontaneous mammary carcinomas (TUBO cells)
- Immunization of Balb/c mice against rat Her-2/*neu* using allogeneic (N202.1A) cells
- Characterization of the role of p185^{neu} protein in tumor and metastasis growth
- Inefficacy of p185^{neu} peptide vaccination in protecting against TUBO cell challenge

8 REPORTABLE OUTCOMES

#1 Production of Her-2/*neu* transgenic mice. One of the major difficulties in studying spontaneous tumors is to get hold of enough transgenic mice for immunization-protection experiments. This problem is made worse by the non-availability of commercial mice carrying the rat *neu* transgene on a well-studied MHC background. Therefore, this project started when a transgenic founder CD1 random-bred breeder male mouse (#1330) carrying the mutated and transforming rat Her-2/*neu* oncogene driven by the mouse Mammary Tumor Virus promoter (NeuT) (kindly made available by Dr. L. Clerici, Euratom, Ispra, Italy) (20) was mated with inbred Balb/c females from Charles River (CR), Calco, Italy. The progeny was screened for the transgene by PCR, and transgene-carrying males were backcrossed with Balb/c CR females for more than 18 generations. These mice now carry the rat NeuT oncogene on the inbred Balb/c genome, and do not react against nor simulate Balb/c CR mice in mixed lymphocyte culture (MLC). Skin transplants from these mice are not rejected by Balb/c CR mice, nor are Balb/c skin transplants are rejected by these mice. These Balb-NeuT mice carry the Her-2/*neu* oncogene in heterozygosis. To secure enough mice for the experiments of this project, our Balb-NeuT transgenic mice are currently bred in: 1) a special breeding unit that has just been set up for this purpose at the Dept. Clin. Biol. Sci., Orbassano; 2) The animal facility of the Inst. Microbiol., Univ of Torino.

#2. A safety back-up breeding colony in isolators at Charles River, Calco Italy has been established. Our Balb-NeuT mice were put in specific pathogen free (SPF) conditions through the transplant of pregnant uterus. This SPF colony is expanding and we plan to use these mice to progressively replace conventional Balb-NeuT mice. The advantages are evident: these mice are

healthier, more uniform and professionally controlled. They could be shipped all over for joint international work focussed on the issues of this program. Next year (1999/2000) cryopreserved Balb-NeuN male embryos will be produced and maintained at Charles River, Calco, Italy.

9 CONCLUSIONS

The experimental work is going on very actively and according to our timetable.

Task 1. Treatment of Balb-NeuT and FVB-NeuN mice with IL-12 (months 1-6). The results obtained are impressive and suggest that stimulation of nonspecific immunity can prevent tumor formation, a provocative deduction. The resemblance of Her-2/*neu* carcinogenesis to that of human beings indicates that IL-12 regimens could serve as a preventive agent. IL-12 appears to inhibit carcinogenesis by slowing down the transition from preneoplastic to overt tumors, halting angiogenesis and activating tumor associated leukocytes through the induction of several secondary cytokines and mediators. Nonspecific immunity can probably never lead to tumor eradication, particularly because some of its effector mechanisms, including its antiangiogenic effects, are cytostatic rather than cytotoxic. They certainly delay the appearance of tumors and in some human situations this could almost be regarded as equivalent to a cure.

Task 2-3. Vaccination of Balb-NeuT (months 3-18) and FVN-NeuN (months 6-22) mice with cytokines-transduced cells. We now have various cell lines expressing rat p185^{neu} protein engineered to release cytokines and hope to be able to estimate their ability to hamper Her-2/*neu* carcinogenesis in the planned times. The production of suitable transfectants has proved more difficult than we expected.

Task 4. Vaccination of Balb-NeuT mice with rat p185^{neu} peptides (Months 13-28). We have already obtained several preliminary data and expect to fully accomplish this Task in the planned time. The preliminary data with transplantable tumors expressing p185^{neu} protein are not encouraging. However, these peptides and their combinations with cytokines may be effective in the early phases of Her-2/*neu* carcinogenesis.

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**Appendix #1. Boggio et al., Interleukin 12-mediated prevention of spontaneous mammary carcinomas in two lines of Her-2/*neu* transgenic mice.
J. Exp. Med. 188:589,1998**

Interleukin 12-mediated Prevention of Spontaneous Mammary Adenocarcinomas in Two Lines of Her-2/*neu* Transgenic Mice

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Summary

The ability of interleukin (IL)-12 to prevent tumors when administered to individuals with a genetic risk of cancer was studied in two lines of transgenic mice expressing rat HER-2/*neu* oncogene in the mammary gland. Female BALB/c (H-2^d) mice carrying the activated HER-2/*neu* oncogene 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 10 mammary glands display invasive carcinomas. Adult FVB mice (H-2^b) carrying the HER-2/*neu* protooncogene develop mammary carcinomas with a longer latency (38–49 wk) and a lower multiplicity (mean of 2.6 tumors/mice). Treatment with IL-12 (5 daily intraperitoneal injections, 1 wk on, 3 wk off; the first course with 50 ng IL-12/day, the second with 100 ng IL-12/day) begun at 2 wk of age in BALB/c mice and at 21 wk of age in FVB mice markedly delayed tumor onset and reduced tumor multiplicity. Analogous results were obtained in immunocompetent and permanently CD8⁺ T lymphocyte-depleted mice. In both transgenic lines, tumor inhibition was associated with mammary infiltration of reactive cells, production of cytokines and inducible nitric oxide synthase, and reduction in microvessel number, in combination with a high degree of hemorrhagic necrosis.

Key words: interleukin 12 • adenocarcinomas • tumor prevention • angiogenesis • Her-2/*neu*

Current genetic studies are leading to the identification of gene mutations that predispose to cancer. They thus hold out the hope that not-yet affected patients or "unpatients" with a defined genetic prognosis can be detected (1). This, indeed, is already a reality in breast cancer, one of the most common malignancies in women.

With the paucity of effective preventive options, probing of the human genome is raising new and dramatic ethical, psychological, and cultural issues (1). On the other hand, identification of a mutated gene and its altered or amplified products is providing a new chance of undertaking immunologic maneuvers against oncogene products (2) in an unprecedented setting, where they may be able to

prevent the onset or inhibit the initial growth of tumors in healthy individuals with a high risk of cancer.

This paper offers evidence of the efficacy of administration of mouse recombinant IL-12 (rIL-12) in counteracting the occurrence and progression of spontaneous mammary carcinomas in the females of two lines of inbred mice transgenic for the rat HER-2/*neu* oncogene under the transcriptional control of mouse mammary tumor virus (MMTV)¹ LTR (3). BALB-NeuT female mice are from a new line of transgenic BALB/c mice carrying the activated HER-2/*neu* oncogene (4) and presenting high mammary

¹Abbreviations used in this paper: iNOS, inducible NO synthase; IP-10, IFN- γ -inducible protein 10; NO, nitric oxide; MIG, monokine induced by γ -IFN; MMTV, mouse mammary tumor virus; MSA, mouse serum albumin; VCAM, vascular cell adhesion molecule.

This work is dedicated to the memory of Giorgio Prodi, M.D., Ph.D., ten years after his untimely death.

tumor multiplicity and relatively fast tumor growth (5). FVB-NeuN females carry the HER-2/*neu* protooncogene (6) and display lower multiplicity and longer latency. Prolonged administration of low doses of rIL-12 delayed tumor onset and reduced tumor multiplicity in both lines. Analogous results were obtained in BALB-NeuT mice permanently depleted of CD8⁺ T lymphocytes.

Tumor inhibition in both lines was associated with deficient peri- and intratumoral angiogenesis, infiltration of reactive cells, production of proinflammatory cytokines, and inducible nitric oxide synthase (iNOS) activation. Vascular damage resulted in a high degree of hemorrhagic necrosis of established tumor masses.

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 MMTV promoter (Tg-NeuT, provided by Dr. L. Clerici, Euratom, Ispra, Italy; reference 5) 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 12 generations and HER-2/*neu* + BALB/c mice (BALB-NeuT) were used in these experiments. Parental FVB-NeuN N202 transgenic mice (6) carrying the rat HER-2/*neu* protooncogene driven by the MMTV promoter on the H-2^b FVB inbred background were provided by Dr. W.J. Muller (McMaster University, Hamilton, Ontario, Canada) and bred in our animal facilities. Females of both transgenic lines show a MMTV-driven overexpression of the transgene in the mammary gland and a definite tumor growth involving the mammary gland epithelium (5-7). Individually tagged virgin females were used in this study. Starting at the age of 5 wk, their mammary glands were inspected once a week, and masses were measured with calipers in the two perpendicular diameters (8). Progressively growing masses >3 mm mean diameter were regarded as tumors. BALB-NeuT mice were killed at 61 wk when these masses were evident in all 10 mammary glands. FVB-NeuN mice were killed when a mammary mass exceeded 2 cm mean diameter, and surviving mice were killed at 61 wk. All mice were evaluated histologically for mammary tumor development and toxicity related to IL-12 administration.

IL-12 Administration. rIL-12 (Genetics Institute, Cambridge, MA) in HBSS supplemented with 0.01% mouse serum albumin (MSA; Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally. MSA control mice received similar injections of MSA only.

Depletion of CD8⁺ Lymphocytes. After thymectomy, a few BALB-NeuT neonatal mice were thymectomized and received 200 µg of purified anti-CD8⁺ cell mAb 53.6.72 hybridoma, anti-Ly2; American Type Culture Collection, Rockville, MD) injected intraperitoneally 2 d later. Complete removal of thymus

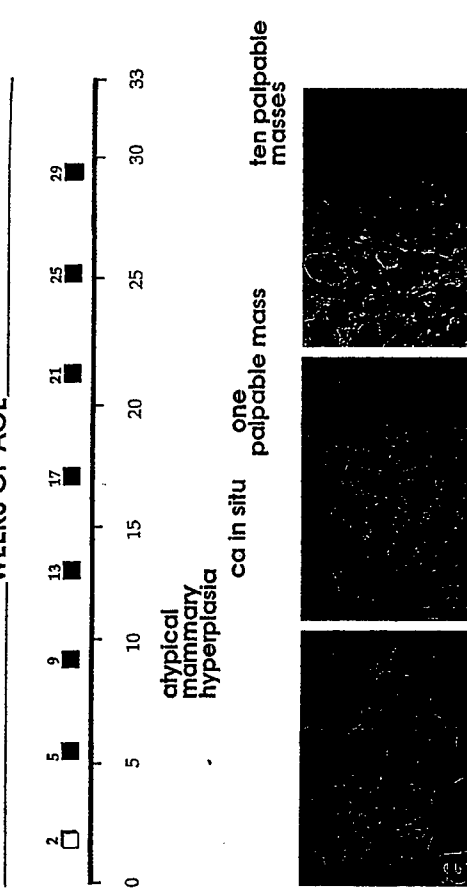
lobes and effective CD8⁺ T lymphocyte depletion was checked at 33 wk. Immunofluorescence of lymph node cells was performed by staining with FITC-conjugated rat and mouse CD3 (clone 145-2C11), CD4 (clone RM4-4), or CD8 (clone 53-6.72) mAbs (all from Pharmingen, San Diego, CA). Cytofluorometry showed a persistent reduction and depletion of CD8⁺ lymphocytes (<4%) compared with untreated mice.

Morphologic Analysis. Groups of two or three BALB-NeuT mice were killed at wk 2 and 3 and then every other week until wk 33; similar groups of FVB-NeuN were killed every 4 wk from wk 5 to 61. 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 immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with anti-CD4, anti-CD8a (all from Sera-Lab, Crawley Down, Sussex, UK), anti-Mac-1 (anti CD11b/CD18), anti-Mac-3, and anti-Ia (all from Boehringer Mannheim, Milan, Italy), antipolyomorphonuclear leukocytes (RB6-8C5; provided by Dr. R.L. Coffman, DNAX Inc., Palo Alto, CA), antitendothelial cells (MEC-13.324; reference 9), anti-IL-1α (Genzyme Corp., Cambridge, MA), anti-TNF-α (Im-muno Contact, Frankfurt, Germany), anti-IFN-γ (10), anti-IL-6 (PharMingen), and anti-iNOS (Transduction Laboratories, Lexington, KY) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat IgG (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 three pathologists in a blind fashion. From mice with multiple tumors, 2 or more samples (1/tumor growth area) and 10 randomly chosen fields in each sample were evaluated for each point determination. For microvessel and cell counts, individual microvessels and cells were counted under a microscope (×400 field (×40 objective and ×10 ocular lens; 0.180 mm² per field). The expression of adhesion molecules, cytokines, and mediators was defined as absent (-) or scarcely (+/-), moderately (+), or frequently (++) present on cryostat sections tested with the corresponding antibodies.

mRNA for Cytokine. Total RNA was prepared from carcinoma masses of mice treated or not with rIL-12 by using Ultraspec (Biotec Lab. Inc., Houston, TX). 2 µg 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 was tested for the presence of murine glucose 3-phosphate dehydrogenase, IL-1α, IL-6, IFN-γ, TNF-α, GM-CSF, monokine induced by γ-IFN (MIG), and IFN-γ-inducible protein (IP-10) sequences in 1 µl reactions (Gene Amp Kit; Perkin Elmer Cetus, Norwalk, CT) performed in 50 µl volumes, by using specific primer pairs prepared by us (IP-10 and MIG) or from Clontech (Palo Alto, CA). The results are arbitrarily scored from - to +++ based on the intensity of UV fluorescence of the ethidium bromide-stained gels as independently evaluated by two operators in a blind fashion.

A BALB-NeuT female mice

WEEKS OF AGE



B FVB-NeuN female mice

WEEKS OF AGE

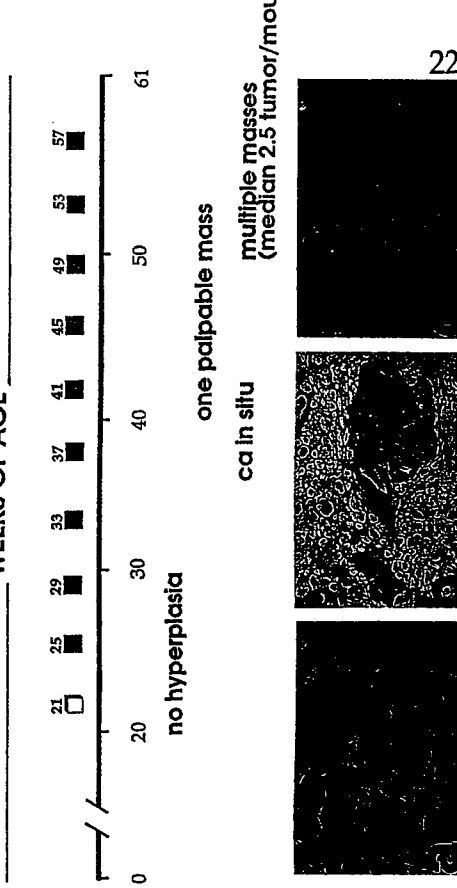


Figure 1. Progression of HER-2/*neu* carcinogenesis in untreated BALB-NeuT and FVB-NeuN female mice. (A) Successive alterations of the mammary gland in untreated BALB-NeuT mice and indications of the time of 5-d courses of rIL-12 administration (□, 50 ng/day; ■, 100 ng/day). Histology shows that ductal atypical hyperplasia (a) is already evident and widely distributed in all mammary glands at 3 wk of age. This hyperplasia progresses to carcinoma in situ (b) between wk 13 and 17, and then to an invading lobular carcinoma (c). (B) Successive alterations of the mammary gland in FVB-NeuN mice during the 61-wk follow-up. Squares (□, ■, as in A). Histology shows that the mammary glands are normal (a) until at least wk 29 of age. Thereafter the neoplastic process begins in one mammary gland with focal hyperplasia and carcinoma in situ (b) and progresses by giving rise to an invasive lobular carcinoma (c) between wk 39 and 48. Original magnification: ×630.

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.

Results

At week 33, lobular carcinomas were palpable in all 10 mammary glands of BALB-NeuT females carrying the activated HER-2/*neu* oncogene (Fig. 1A). FVB-NeuN females carrying the HER-2/*neu* protooncogene display one or more mammary glands with tumor at 49 wk, and the mean number affected at 61 wk is still very low (Fig. 1B). This rapid onset and total gland involvement suggests that expression of activated HER-2/*neu* in BALB-NeuT mice requires few, if any, additional genetic events to transform the mammary epithelial cell (7), whereas the delayed onset and asynchronous progression observed in the other line indicates that overexpression of HER-2/*neu* protooncogene results in stochastic tumor development.

These kinetic patterns provide two distinct models of HER-2/*neu* mammary oncogenesis with which to test the effect of prolonged administration of low doses of rIL-12. Since histologic examination revealed that widespread atypical hyperplasia was already evident in the mammary glands of 3-wk-old BALB-NeuT mice, and HER-2/*neu* mammary oncogenesis quickly progressed to overt carcinoma, these mice first received a 5-d course of 50 ng of rIL-12 plus MSA, injected intraperitoneally at 2 wk of age. The dose was then increased to 100 ng, and 5-d courses followed by 3 wk off were run from wk 5 to 29 (Fig. 1A). MSA control mice received similar courses of MSA only. Mice were inspected weekly. Those displaying progressively growing masses in all 10 mammary glands and those that reached 33 wk of age were killed and morphologically examined.

A significant delay in the onset of the first mammary tumor was evident in the treated mice as compared with the MSA controls. Moreover, at wk 33 the number of mammary glands with a palpable tumor was much lower (Fig. 2, top). Histologic examination of the mammary glands at progressive time points in a fashion blind to the treatment revealed a general delay in the progression of the HER-2/*neu* oncogenesis in the treated mice. Atypical hyperplasia was less vigorous and less widely distributed than in the MSA controls, and no carcinoma *in situ* was found before wk 15 (data not shown). This delay in tumor progression was associated with deficient neovascularization and a marked increase in infiltrating CD8⁺ and, to a lesser extent CD4⁺ lymphocytes (Table 1). Lymphocyte infiltration was concomitant with enhanced vascular cell adhesion molecule (VCAM)-1 expression (Fig. 3), as well as expression of mRNA for proinflammatory cytokines and their production (Table 1). Extensive expression of iNOS on tumors from treated mice was shown immunohistochemically (Fig. 3). This suggests that IL-12-primed NO exerted a cytotoxic and antitumor activity directly or through its reactive products (11, 12). Moreover, strong expression of IP-10

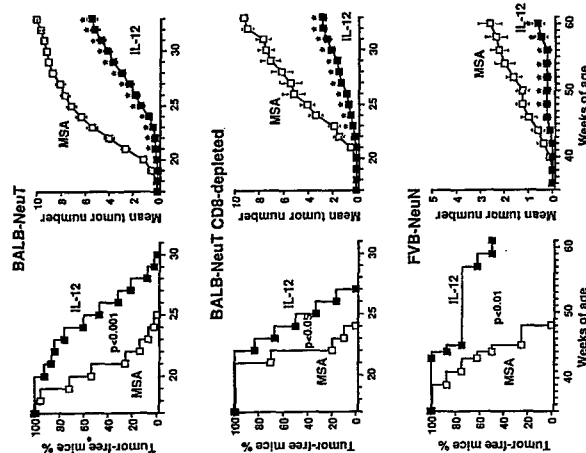


Figure 2. Inhibition of mammary carcinogenesis in HER-2/*neu* transgenic female mice treated with rIL-12. Percentage of tumor-free mice and mean number of palpable mammary carcinomas per mouse calculated as cumulative number of incident tumors/total number of BALB-NeuT mice, BALB-NeuT mice CD8⁺ T lymphocyte depleted by neonatal thymectomy and anti-CD8⁺ mAb treatment, and FVB-NeuN mice treated with rIL-12 (■) or MSA only (□). Stars (*) denote time points at which tumor multiplicity was significantly different ($P < 0.05$ at least) between MSA and rIL-12 treatment groups. Group sizes were: BALB-NeuT, 38 MSA control mice and 43 rIL-12-treated mice; thymectomized and anti-CD8⁺ mAb treated BALB-NeuT mice, 10 MSA control mice, 6 rIL-12-treated mice; FVB-NeuN, 8 mice each group.

and MIG mRNA was revealed by reverse transcriptase PCR in the tumor area from treated mice (Table 1). These two chemokines mediate the angiogenic activity of IL-12, and, in combination, chemoattract activated T cells (13-15). Their modulation is probably a consequence of IL-12-induced expression of IFN- γ and TNF- α in NK and T cells, IFN- γ and TNF- α also amplify the production of proinflammatory cytokines, angiogenic inhibitors, and NO by reactive cells (15). This multifactorial scenario offers an explanation for both the inhibition of tumor neovascularization and vascular damage leading to the impressive ischemic and hemorrhagic necrosis of advanced carcinomas in rIL-12-treated BALB-NeuT mice (Fig. 4).

Our previous studies have shown that CD8⁺ lymphocytes play a pivotal role in the antitumor reaction elicited by IL-12, whereas CD4⁺ cells play an inhibitory role (8, 16, 17). To evaluate the weight of CD8⁺ cells in rIL-12-delayed oncogenesis, BALB-NeuT mice treated with rIL-12 or MSA only were thymectomized at 4 wk and received

Table 1. Effects of rIL-12 Treatment on Neovascularization, the Expression of Adhesion Molecules, the Secretion of Mediators, and the Infiltration by Reactive Cells in 7-mm Mean Diameter Tumors Growing in BALB-NeuT and FVB-NeuN Female Mice

Pathologic findings (immunohistochemistry)	BALB-NeuT mice			FVB-NeuN mice		
	MSA	MSA + IL-12		MSA	MSA + IL-12	
Microvessel count	15.9 $\pm$ 2.1	9.5 $\pm$ 1.3*		19.7 $\pm$ 3.7	10.5 $\pm$ 2.4*	
Endothelial adhesion molecules						
ICAM-1	+	+		+	+	+
VCAM-1	-	++		-	++	++
ELAM-1	-	+/-		-	-	-
Infiltrating cells						
Macrophages	12.5 $\pm$ 4.1	13.4 $\pm$ 3.8		16.1 $\pm$ 4.2	14.2 $\pm$ 5.2	
Granulocytes	5.2 $\pm$ 1.9	7.6 $\pm$ 2.3		5.0 $\pm$ 2.8	6.9 $\pm$ 3.0	
CD8 ⁺ lymphocytes	2.9 $\pm$ 0.8	42.0 $\pm$ 8.3*		4.8 $\pm$ 2.1	14.6 $\pm$ 6.1*	
CD4 ⁺ lymphocytes	3.1 $\pm$ 1.7	15.2 $\pm$ 5.1*		3.6 $\pm$ 1.9	5.7 $\pm$ 2.6	
Cytokines and mediators						
IL-1 β	-	+/-		-	+/-	+/-
TNF- α	+/-	+		+/-	+	+
IFN- γ	-	+		-	+	+
IL-6	+/-	+		+/-	+	+
iNOS	-	++		-	++	++
Reverse transcriptase PCR (mRNA)						
Cytokines and chemokines						
IL-1 β	++	+++		NT†	NT	NT
TNF- α	-	+		NT	NT	NT
IFN- γ	-	+/-		NT	NT	NT
IL-6	++	++		NT	NT	NT
GM-CSF	+/-	+		NT	NT	NT
MIG	+	++		NT	NT	NT
IP-10	+	++		NT	NT	NT

Microvessel counts were performed on cryostat sections tested with an antiendothelial (CD31) mAb as described in Materials and Methods. The expression of adhesion molecules, cytokines, and mediators on cryostat sections was probed with the corresponding mAb. From mice with multiple tumors, two or more samples (one sample per tumor) were evaluated. At least 10 fields were counted per sample. Values are expressed as mean $\pm$ SD of five BALB-NeuT and four FVB-NeuN mice. An arbitrary score (from - to +++) was used to indicate the amount of detected signal in a semiquantitative reverse transcriptase PCR analysis, as described in Materials and Methods. At least three independent tumors from BALB-NeuT mice were evaluated, and a representative expression pattern is shown.

*Value significantly different ($P < 0.001$) than that in MSA-treated mice.

†NT, not tested.

200 μ g of anti-CD8 mAb intraperitoneally. Both the progression of mammary oncogenesis and the protective effect of IL-12 were unaffected by this permanent CD8⁺ depletion (Fig. 2, middle).

The protective effect of rIL-12 was also evident in FVB-NeuN mice. Here, because of the slower tumor progression and the virtual absence of atypical mammary hyperplasia foci before wk 29, the initial 5-d course was administered to 21-wk-old mice (Fig. 1B), followed by the same schedule as for the BALB-NeuT mice until wk 57. Mice displaying a tumor mass > 2 cm mean diameter and those that reached the wk 61 of age were killed: 50% of the treated

mice were tumor-free, whereas all of the MSA controls displayed palpable tumors. The mean number of mammary glands with a palpable tumor was 2.6 per mouse in the MSA controls compared with only 0.9 in the IL-12-treated mice (Fig. 2, bottom). Deficient peri- and intratumoral vascularization, enhanced expression of adhesion molecules, enhanced CD8⁺ infiltration, cytokine production, and iNOS activation (Table 1 and Fig. 5), and large areas of hemorrhagic necrosis in established tumors (Fig. 4) were evident as in the IL-12-treated BALB-NeuT mice.

Increased spleen and liver extramedullary hematopoiesis and a slight exacerbation of hepatic periportal mononuclear

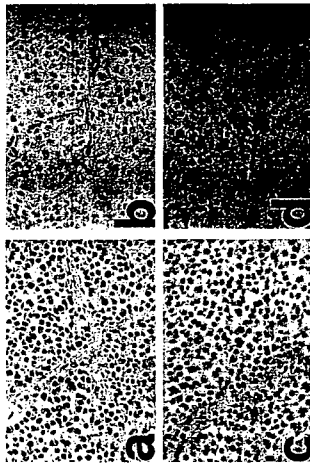


Figure 3. Cryostat sections of invading lobular carcinomas in MSA control (a and b) and IL-12-treated (c and d) BALB-NeuT mice. VCAM-1 is absent in tumor blood vessels of MSA control mice (a), whereas it is expressed in those of rIL-12-treated mice (c) as revealed by anti-VCAM-1 mAb. In the MSA control tumors, iNOS expression is almost undetectable in macrophages (b), whereas in rIL-12-treated mice it is clearly evident in macrophages intermingled among tumor cells (d) as shown by anti-iNOS mAb. Observations made in 7-mm mean diameter tumors. Similar patterns are evident in all established tumors. Original magnification: X630.

infiltration were the only signs of chronic rIL-12-related lesions in both lines of mice, showing that this cytokine is effective at well-tolerated doses.

Discussion

rIL-12 administration in young BALB-NeuT mice carrying the activated Her-2/*neu* oncogene delays the appearance of tumor and reduces the number of mammary glands involved. In adult FVB-NeuN mice carrying the HER-2/

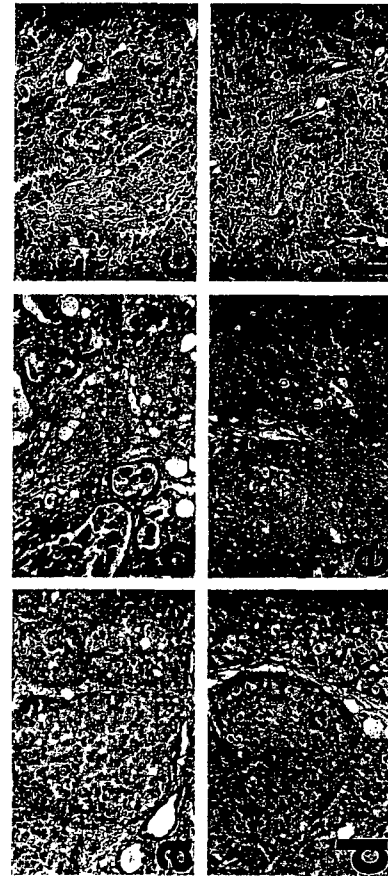


Figure 4. Histologic features of invading lobular carcinomas in BALB-NeuT (a) and FVB-NeuN (b) MSA control mice and in rIL-12-treated BALB-NeuT (c and d) and FVB-NeuN (e and f) mice. The impressive ischemic and hemorrhagic necrosis disaggregating the neoplastic masses becomes evident from wk 27 in BALB-NeuT (c) and wk 53 in FVB-NeuN (e) mice. In both cases it eventually results in extensive tumor destruction (e and f). Original magnification: X630.

mized BALB-NeuT mice suggest that CD8⁺ lymphocytes do not play a major role here, even if they are the dominant population of reactive cells infiltrating the tumor area in rIL-12-treated mice. It is possible that NK cells are the leading actors in this IL-12-provoked delay in oncogenesis (18). Since the NK1.1 mAb recognizes an allelic form of an NK cell antigen expressed by C57BL/6 but not BALB/c mice, the type and function of lymphocytes first involved are currently under investigation in (BALB/c × C57BL/6) NeuT⁺ F1 mice.

The nonessential role of cytotoxic CD8⁺ lymphocytes *in vivo* fits in well with both the marginal cytotoxic activity and the lymphokine release observed when T lymphocytes from rIL-12-treated mice were restimulated *in vitro* with APCs pulsed with the product of the rat HER-2/*neu* oncogene (p185), a self-protein in these transgenic mice (data not shown). These findings, along with morphologic data, rather suggest that rIL-12 essentially protects by halting angiogenesis (19) through the induction of several secondary cytokines and mediators, thus slowing down the transition from hyperplasia to overt carcinoma (20, 21). Moreover, the secondary cytokines and the tertiary chemokines and monokines elicited by IL-12 are apparently responsible for later vessel endothelial wall injury and hemorrhagic necrosis of established tumors (12, 13, 22–24). The endothelial vessel alteration and hemorrhagic necrosis observed in both lines of rIL-12-treated mice are reminiscent of those observed during rejection of an established transplantable mammary adenocarcinoma in BALB/c mice treated with systemic rIL-12 or adenocarcinoma cells engineered to release IL-12 (8).

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Whether immunological maneuvers in healthy individuals at risk prevent the development of cancer is a question that has rarely been examined. Most studies have evaluated immunization before transplantation with frank tumor cells. Immunoprevention of cancer has been attempted in a few models of chemical carcinogenesis. Interpretation of these experiments may be confounded by the fact that carcinogens may be immunosuppressive.

Noguchi et al. have shown that similar doses of IL-12 inhibit 3-methylcholanthrene carcinogenesis in mice through the release of cytokines and mediators and nonspecific immune mechanisms (25). Our findings extend these observations by underscoring the potential of nonspecific immune mechanisms and their cross-talk with the endothelial cells of tumor vessels in the inhibition of HER-2/*neu* oncogenesis.

The HER-2/*neu* oncogene is expressed in a substantial proportion of human mammary carcinomas and its product seems to be a promising target for specific immune intervention (26). However, its expression in humans is not the same as the expression of a xenogenic oncogene in transgenic mice, and this issue may somewhat impact specific immunization rather than nonspecific immune mechanisms. The close resemblance of the progression of mammary carcinogenesis in HER-2/*neu* transgenic mice to that in women suggests that chronic administration of nontoxic rIL-12 regimens may be a significant prophylactic strategy. The direct proportionality between the length of carcinogenesis progression and the efficacy of rIL-12 suggest that a "soft" immunologic alternative to controversial and distasteful mastectomy may be envisaged.

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**Appendix #2. Di Carlo et al., Analysis of mammary carcinoma onset
and progression in HER-2/*neu* oncogene transgenic mice
Lab. Inv., 1999 in press**

Di Carlo

**ANALYSIS OF MAMMARY CARCINOMA ONSET AND
PROGRESSION IN HER-2/*neu* ONCOGENE TRANSGENIC
MICE REVEALS A LOBULAR ORIGIN**

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Short running head: Lobular mammary carcinoma in transgenic
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Di Carlo

Di Carlo

Abstract

Morphological 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 (PCNA) immunostaining, and coincides with the activation and maximal extension of tumor angiogenic process as assessed by microvessel count (anti-CD31), anti-b3 integrin and anti-laminin immunostaining. Neovascularization is accompanied by vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) production by hyperplastic epithelial cells.

By contrast with the BALB-NeuT tumors, E-cadherin expression is almost nil 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.

Abbreviations

BALB-NeuT, BALB/c (H-2^d) mice carrying the activated rat HER-2/neu oncogene; bFGF, basic fibroblast growth factor; ERs, estrogen receptors; FVB-NeuN, FVB (H-2^q) mice carrying the wild-type rat HER-2/neu proto-oncogene; MMTV, mouse mammary tumor virus; PCNA, proliferating cell nuclear antigen; PRs, progesterone receptors; VEGF, vascular endothelial cell growth factor.

Keywords:

HER-2/neu oncogene
Mouse mammary carcinoma
Lobular carcinoma
Tumor angiogenesis
Transgenic mice

Introduction

World-wide, breast cancer is the most frequent malignancy of woman (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, since they are almost exclusively virus and/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 carcinogenesis 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 carcinogenesis. Its amplification and overexpression, in fact, have been observed in a large percentage of primary human breast cancers and appear to be inversely correlated with survival (King et al, 1985; Slamon et al, 1987; Slamon et al, 1989; Di Giovanna et al, 1996), 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 histological and pathological 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 paper we report that BALB/c transgenic female mice carrying the activated rat HER-2/neu oncogene (Muller et al, 1988; Boggio et al, 1998) 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 tumors are similar to the alveolar-type human lobular mammary carcinoma.

Inclusion of a lobular type in the histological classification of rodent mammary tumors (Russo and Russo, 1996) makes it more detailed and fully comparable to that of human forms (Rosai, 1996).

Here 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.

Di Carlo

Results

Histological 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 wks of age. They then began to develop multiple mammary tumors that progressively involved all ten glands by the 33rd wk. 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^d) mice carrying the HER-2/neu proto-oncogene with HER-2/neu product overexpression developed mammary carcinomas with a longer latency (38th-49th wk) 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 wks (Fig. 1,a) and characterised 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 wk, the ductules and acini within the lobules were distended by the solid, occlusive growth of this epithelial cell population (Fig. 1,b). 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 wk, alveolar groups of neoplastic cells with no myoepithelial lining infiltrated the surrounding adipose tissue (Fig. 1,c). 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 35-37 wks, 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.

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,d). Occasionally in BALB-NeuT and frequently in FVB-NeuN mice they had darker cytoplasm 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. 2,c).

Immunohistochemistry

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

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

Studies were also performed in BALB transgenic mice to investigate angiogenesis during tumorigenesis. Before (2nd wk) and during hyperplasia (5th wk), and when lobular carcinoma in situ (15th wk) and invasive alveolar lobular carcinoma (28th wk) developed, tissue specimens were tested with anti-endothelial cells (CD31), anti-basement membrane components (anti-laminin and anti-collagen type IV) and anti-b3 chain antibody, which recognises the adhesion receptor $\alpha_v\beta_3$ selectively expressed on growing vessels.

Microvessel counts indicated that hyperplastic foci were much more vascularized than non-hyperplastic or carcinomatous tissue (Table 1 and Fig. 4,a,b). Several capillary sprouts in hyperplastic foci expressed the β_3 chain of the $\alpha_v\beta_3$ receptor (Fig. 4,c) which was absent in normal

Di Carlo

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 VEGF and bFGF, absent or scarcely present in normal mammary tissue, were clearly expressed by epithelial cells during hyperplasia (Fig. 4, e 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. 4, f).

Detection of Estrogen and Progesterone receptors (ERs and PRs)

A low level of ERs (8.1 fmol; range 7.4 to 9.3) and PRs (16.2 fmol/mg cytosol protein; range 14.8 to 17.7) was found in BALB-NeuT carcinomatous tissue. The Kd values for both receptors ranged from 0.1 to 0.2 nM.

RT-PCR analysis

Expression of bFGF and VEGF was also demonstrated by RT-PCR 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 wild type HER-2/neu oncogene develop mammary carcinomas asynchronously with a longer latency and lower multiplicity (Muller et al, 1988; Boggio et al, 1998). In spite of these kinetic differences, histological 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 protooncogene is frequently activationally mutated in FVB-NeuN transgenic mice (Siegel et al, 1994).

Di Carlo

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 histological 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 characterised 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. This histologic and ultrastructural findings are identical to those observed in human mammary lobular carcinoma (Rosen and Oberman, 1993; Murad, 1971; Eusebi et al, 1977; Nesland et al, 1985). 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 have probably not defined these tumors as lobular (Munn et al, 1995; Bouchard et al, 1989; Guy et al, 1996) because they mainly focused on the cytological aspects of transformed epithelial cells which were identified as "intermediate cells" (Cardiff et al, 1991) i.e. clear or basal cells supposed to originate from a metaplastic alteration occurring in mammary epithelial and/or myoepithelial cells. Furthermore, the tendency of tumor cells to grow in a solid, loosely cohesive manner may be due 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). Certainly, 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 (25-40%), whereas in lobular carcinoma this association is rarely found (1-13%) (Porter et al, 1991; Zschiesche et al, 1997; Querzoli et al, 1998).

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 ERs and PRs. 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 ERs and PRs were found in 12 patients with the alveolar variant of invasive lobular carcinoma, though values ranged from > 300

Di Carlo

to 1,495 fmol/mg 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 is frequently reduced in human mammary lobular carcinomas (Vos et al, 1997) mainly in those with a more pronounced metastatic potential (Siitonen et al, 1996; Berx et al, 1995). 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 (LA) units or terminal end buds (TEB), which are regarded as equivalent to the terminal ductal lobular units (TDLU) of the female human breast (Russo and Russo, 1996; Cardiff, 1998).

There is evidence that spontaneous and chemically induced ductal tumors develop in LA/TEB units (Russo and Russo, 1996; Cardiff, 1998). Since these units contain the proliferative stem cell populations most sensitive to the effects of somatic cell mutation, they appear to be the site of origin for most mammary cancers, including those of lobular type. It has been hypothesised that human lobular carcinoma arises from a more complex and differentiated lobular structure (lobule type 2) that evolves from TDLU (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 LA/TEB 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, characterised 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 β_3 subunit of $\alpha_v\beta_3$ integrin, which has

been reported to promote endothelial cell migration, angiogenesis and protection from apoptosis (Shattil, 1995; Brooks et al, 1994). Its expression identifies new vessel sprouts and is a real indicator of neovascularization (Brooks et al, 1994).

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 immobilised in the extracellular matrix are normally inactive because of their strong adherence to heparin sulphate proteoglycans (Rak and Kerbel, 1997; Czubyko et al, 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 (Russo and Russo, 1996; Munn et al, 1995), while 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.

Methods

Mice

A transgenic CD1 random-bred breeder male mouse (no. 1330) 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 back crossed 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 protooncogene driven by the MMTV promoter on the H-2^k FVB inbred background

Di Carlo

were provided by Dr. W.J. Muller (McMaster University, Hamilton, Ontario, Canada) and bred in our animal facilities. Females of both lines show a MMTV-driven overexpression of the transgene in the mammary gland and a definite tumor growth involving its epithelium (Lucchini et al, 1992; Guy et al, 1992; Guy et al, 1996). Individually tagged virgin females were used in this study. Starting at the age of 5 wk, 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.

Histological and ultrastructural analysis

Groups of two or three BALB-NeuT mice were killed at wk 2 and 3 and then every other week until wk 33 of age; similar groups of FVB-NeuN were killed every 4 wk from 5 to 61 wk of age. For histologic evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μ m, 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 min with anti-endothelial cells (mEC-13.324) (Vecchi et al, 1994), 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) and anti-Neu (C-18) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-proliferating cell nuclear antigen (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 IgG (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 three pathologists in a blind fashion. 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

Di Carlo

determination. For microvessel and reactive cell counts, individual microvessels and cells were counted under a microscope X 400 field (X 40 objective and X 10 ocular lens; 0.180 mm² 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 X 600 field (X 60 objective and 10 ocular lens; 0.120 mm² 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

ERs and PRs were assessed as reported by Carbone and Vecchio (1986) 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 (Kd) 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 (Biotex Lab. Inc., Houston, TX). 2 μ g of RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (200 U) in 50 μ l 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, VEGF and bFGF sequences in PCR reactions (Gene Amp Kit; Perkin Elmer Cetus, Norwalk, CT) performed in 20 μ l volumes and amplified by 30 PCR cycles, by using specific primer pairs prepared by us (VEGF) or from Stratagene (La Jolla, CA) (bFGF).

Di Carlo

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Di Carlo

Di Carlo

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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 $\pm$ 3.2 ^a	15.9 $\pm$ 2.1*
PCNA immunoreactivity rate	65.2 $\pm$ 13.1%	24.8 $\pm$ 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 $\pm$ 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 hyperplasia.

Di Carlo

Fig.1 Histologic features of lobular carcinoma development in rat HER-2/neu transgenic mice (a-d). In 5-wk-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-wk-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 histological 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 is almost completely replaced by a solid occlusive proliferation of a relatively uniform population of round cells with a pale cytoplasm. (Hematoxylin and eosin staining; a, c, d, e, f x 200; b x 100).

Fig.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 x 2750; b x 1900; d x 1450).

Fig.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 (PCNA) 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 x 630).

Fig.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)

Di Carlo

present in hyperplasia (c) express the β_3 subunit of the $\alpha_v\beta_3$ receptor, which is almost absent in lobular carcinoma (d). bFGF is clearly expressed by epithelial cells during hyperplasia (e), while in lobular carcinoma a marked bFGF staining is evident in the extracellular matrix bordering the neoplastic lobular structure (f). (a-f x 630).

- Fig.5 Expression of 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-wk-old mouse. The 292 bp bFGF band is wider than that of VEGF (294 b). G3DPH message (452 bp; lane A) served as control. The marker (lane M) is Hae III digest of Phi x 174.



Fig.3



Fig.4

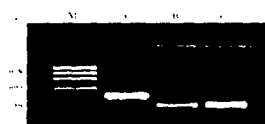


Fig.5



Fig.1

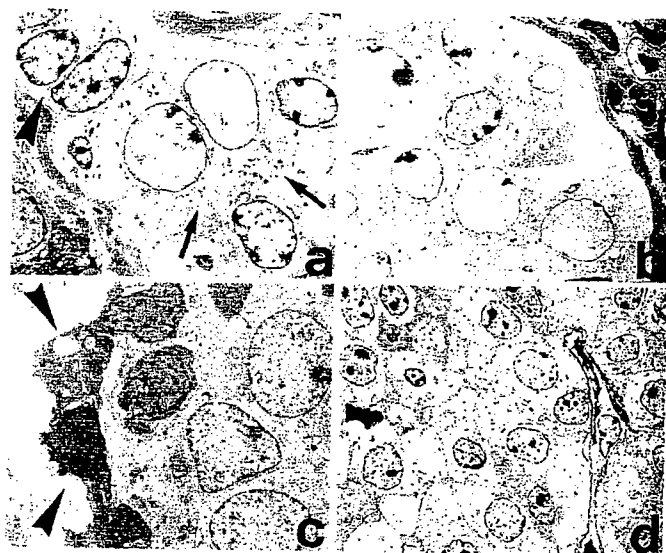


Fig.2

Appendix #3. Lollini and Forni, Specific and non specific immunity in the prevention of spontaneous tumors. *Immunol. Today*, 1999 in press

Summary

It is becoming evident that antitumor vaccines cannot cure established tumors. However, the fact that many antigens are common to distinct tumors holds out the hope of their use to immunize persons at risk. By inhibiting the angiogenic phenotype of pretumoral cells and activating tumor-associated leukocytes cytokines can halt the cancerogenic progression.

Presentation of tumor-associated antigens by dendritic cells has shown that defined peptides can elicit a specific antitumor immunity¹. The immune recognition of multiple dominant and subdominant tumor antigens could be evoked by whole tumor cells engineered with adhesion and costimulatory molecules, suicide genes or cytokines (for a review see ²). When the same tumor is engineered to release different cytokines, is the cytokine released that decides which immune mechanisms are elicited in a privileged way. Thus, a selective activation of those that are the most appropriate to the stages of tumor progression is feasible³.

These vaccine strategies are often effective. Tumors 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}. The result has been to change our way of thinking about the immune control of tumors. However, an usual pitfall in evaluating the potential of these new vaccines is to overlook that abrogation of the tumorigenicity of gene-engineered cells and effective immunization of healthy animals against a subsequent challenge by wild-type tumor cells have little to do with therapy⁴. Their real ability to cure existing tumors has hardly ever been investigated. In most studies only a minority of tumor-bearing mice were cured, and even this limited efficacy was solely achieved when the vaccine was administered in the first few days after the challenge⁵, while corresponding clinical trials have not disclosed any significant ability to cure⁶.

Further consideration must none the less be given to the question of the inability of these vaccines to cure tumors. 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 approachable by these new vaccines (Table I), and the cure of clinically evident tumors has been both 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 explanation of these clinical attempts. Paradoxically, their negative results are often blamed on the poor reliability of the indications provided by 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. Clinical evidence in support of this view, however, is still awaited⁶.

Our point here is that the high degree of immunogenicity 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 tumors. This issue is underscored by the demonstration that these mechanisms lead to the rejection of normal tissues, but not tumors expressing the same target antigen⁷, and by the hundred and one ways in which an established tumor manages to elude them⁸.

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. Even so, the experimental data indicate that cancer vaccines could be able to cope with minimal residual disease, prevent recurrences and inhibit incipient metastases after conventional tumor management⁹.

Tumor prevention by specific immunity

Current studies have led to the discovery of gene mutations that predispose to cancer. It may thus be possible to detect not-yet patients with a defined genetic prognosis¹⁰. 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. Molecular characterization of altered gene products predictably destined to become a tumor antigen will be the first step towards the engineering of effective vaccines to be used for this purpose.

Identification of human tumor antigens is showing that a few of them are expressed by distinct tumors¹¹. If the most common antigens are eventually found to number no more than fifty or so, vaccination of healthy individuals against tumors will begin to seem a much more feasible proposition. Immunologic maneuvers have been clearly shown to prevent the onset of virus-related tumors, such as Marek's disease of poultry¹², and human hepatocellular carcinoma¹³, 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 tumors that most commonly arise in women and men of such a population. A constraint is imposed by the polymorphism of the glycoproteins of the major histocompatibility complex. Different vaccines would need to be prepared in order to fit in the polymorphic peptide-binding clefts. It is predictable that certain tumor 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 elicitation of an efficient immune response offers protection against spontaneous tumors. The central tenet of tumor immunology is that recognition of tumor antigens is followed by the establishment of a long-lasting immune memory and the specific killing of tumor cells. This notion is supported by experimental data from many transplantable tumors. The use of appropriate vaccines has shown that even spontaneous tumors first claimed to be nonimmunogenic¹⁴ can induce protection against a subsequent challenge¹⁵. Nevertheless, very few data are available on spontaneous tumors which display a longer and more complex natural relationship with their host than transplantable forms.

Mice transgenic for oncogenes may form appropriate models to explore the defensive role of the immune system in tumorigenesis. The protection provided by vaccination of mice transgenic for the rat *neu* oncogene with the DNA coding for the extracellular part of the *neu* p185 product¹⁶ and with soluble p185 protein¹⁷ suggests that they hamper the onset of tumors. The challenge is to pass from a proof of principle to an effective human vaccine.

Antigen-loss variants are unlikely to emerge as tumor escape mechanisms when the target molecule is directly linked to neoplastic transformation and progression, as in the case of p185 (in preparation) and other oncogene products. A more probable escape route is offered by the defects in antigen processing and MHC class I down-regulation detected in murine¹⁸ and human carcinomas¹⁹.

Nonspecific immunity strikes back

Blockade of tumor 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) are cured by repeated immunizations with cytokine gene engineered TSA cells^{3, 5} or TSA peptide pulsed dendritic cells¹ begun immediately after the challenge, and almost none when they are begun on day 7, whereas the great majority of these 7-day-old tumors are cured by repeated injections of low doses of interleukin-12^{5, 9}. Tumor destruction results from three major mechanisms:

- a) destruction of tumor vessels by polymorphonuclear leukocytes;
- b) indirect inhibition of angiogenesis by secondary interferon- γ , tumor necrosis factor- α and third-level chemokines;
- c) activation of leukocyte subsets capable of producing proinflammatory cytokines, cytotoxic T lymphocytes and antitumor antibodies⁹.

Surprisingly, similar interleukin-12 triggered mechanisms inhibit both chemical²⁰ and *neu*-oncogene dependent²¹ carcinogenesis. When BALB/c mice were injected subcutaneously with 3-methylcholanthrene, 100 ng interleukin-12 administered systemically 5 days a week for 18 weeks (3 weeks on and 1 week off) delayed tumor appearance and reduced tumor incidence. Secondary interferon- γ , interleukin-10, and tumor necrosis factor- α were induced throughout the treatment. High production of interferon- γ by CD8 T cells and a Th2 $\rightarrow$ Th1 or Th0 shift in the cytokine secretion profile of CD4 T cells were also seen in the treated mice²⁰.

The mammary glands of both female Balb/c mice carrying the activated HER-2/*neu* oncogene and adult FVB female carrying the HER-2/*neu* protooncogene mice progress through atypical hyperplasia to in situ and invasive lobular carcinoma. This progression begins in BALB/c-*neu* mice when they are weaning while in FVB-*neu* mice when they are adults. Systemic treatment of mice with preneoplastic lesions with interleukin-12 5 days a week (1 week on, 3 weeks off; first course 50 ng interleukin-12/day, the remainder 100 ng/day) markedly delayed tumor onset and reduced tumor multiplicity. Analogous results were obtained in immunocompetent and permanently CD8⁺ T lymphocyte-depleted mice. In both transgenic lines, tumor inhibition was associated with mammary infiltration by reactive cells, production of cytokines and iNOS, reduction in microvessel number and a high degree of hemorrhagic necrosis²¹.

These experiments naturally encourage one to suggest that stimulation of nonspecific immunity can prevent tumor formation, an unexpected and indeed provocative deduction. The resemblance of methylcholanthrene and Her-2/*neu* carcinogenesis models to that of human beings indicates that

nontoxic interleukin-12 regimens might constitute a significant prophylactic strategy. Generalization of these findings to other tumors and cytokines could enable stimulation of nonspecific immunity to be used to protect not-yet patients with a genetic risk of cancer (Fig. 1, panel A) and those with preneoplastic lesions (Fig. 1, panel B and C) as a "soft" immunologic alternative to controversial and distasteful preventive manoeuvres¹⁰.

Apparently, interleukin-12 inhibits cancerogenesis by slowing down the transition from preneoplastic to overt tumors, halting angiogenesis and activating tumor associated leukocytes through the induction of several secondary cytokines and mediators^{20,21}. Nonspecific immunity can probably never lead to tumor eradication, particularly because some of its effector mechanisms, including its antiangiogenic effects, are cytostatic rather than cytotoxic. They certainly appear to delay the appearance of tumors and in some human situations this could almost be regarded as equivalent to a cure²². Several chemopreventive agents are currently under investigation, including several new selective estrogen receptor modulators²³. We can envisage a future when combined chemical and immunologic preventive management will significantly decrease the incidence of clinically evident tumors in individuals at risk.

Conclusions

Molecular data on tumor antigens and elucidation of the protective role of the immune system in tumorigenesis may delineate new strategies in oncology. The immunotherapeutic path trodden so far has not much experimental backing and may be too hard to follow. Its therapeutic success has been undeniably marginal. Even so, it was fully justified by the dramatic seriousness of the problem and led to many scientific discoveries. Elaboration of antitumor vaccines and investigation of the defensive role of nonspecific immunity in tumorigenesis will not be easy. It could, however, be rewarded by the creation of effective tumor prevention strategies.

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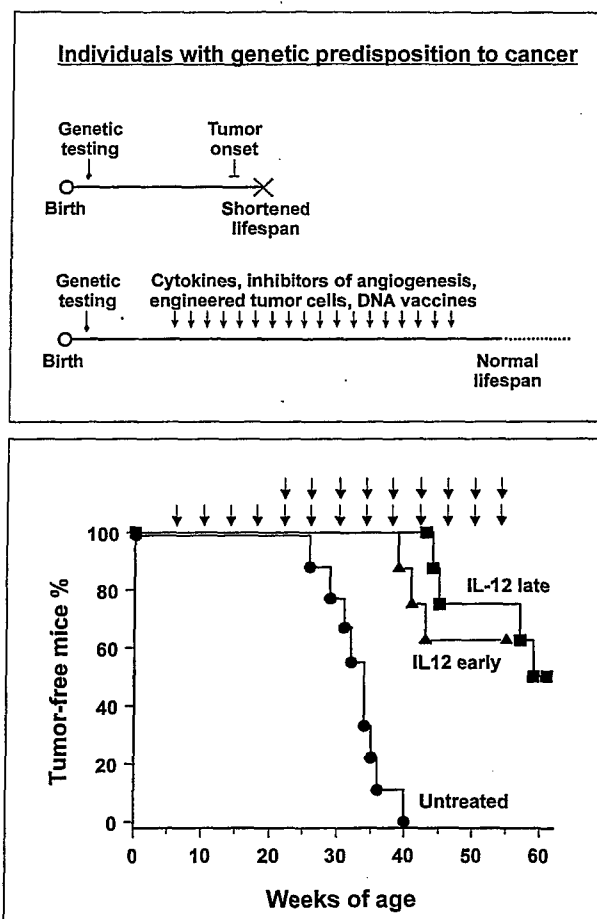
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Legend to Figure 1.

Fig. 1. The tumor prevention modalities currently available to individuals inheriting a genetic predisposition to cancer are distasteful and controversial^{10,12}. We suggest that tumor progression can be interrupted by maneuvers that stimulate nonspecific host immune responses and inhibit tumor angiogenesis^{20,21}.

The graph 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 time. A chronic treatment with rIL-12 (each arrow represents one week of IL-12 treatment, as described in the text and in Ref 21) significantly reduced tumor incidence. Halt of tumor progression was obtained both starting IL-12 treatment both early in life of mice (IL-12 early) and when mice are already adult (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.



**Appendix #4. Boggio et al., Ability of systemic IL-12 to hamper
progressive stages of mammary carcinogenesis in Her2/neu transgenic mice,
(to be submitted)**

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Key words: Interleukin-12; HER2/neu carcinogenesis; tumor prevention; mammary carcinomas

Abstract

Background: Previous studies in mice have shown that chronic administration of recombinant IL-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 genetic risk of cancer or carrying preneoplastic lesions. **Methods:** Starting at progressive stages of mammary carcinogenesis, female BALB/c and FVB mice carrying the activated rat HER2/neu oncogene (BALB-neuT) or the protooncogene (FVB-neuN) under mouse mammary tumor virus promoter received multiple 5-day courses of different doses of IL-12. The times of tumor appearance and multiplicity and the histopathological features of the neoplastic lesions were evaluated. **Results:** In both BALB-neuT and FVB-neuN mice, 5-day courses of 50/100 ng IL-12/day inhibited mammary carcinogenesis when they coincided with the progression of early preneoplastic lesions. Inhibition appears to mostly depend on IL-12's ability to interfere with early tumor angiogenesis. Later treatments are much less effective and daily doses of 10 and 2 ng are useless. **Conclusions:** The efficacy of early IL-12 courses suggests that they could be used to prevent mammary tumors in individual 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. Lifetime administration is not required for genetically determined cancers with a long natural history. Precise understanding of tumor progression means that effective treatments can be commenced relatively late in the life of individuals at risk.

Introduction

The remarkable ability with which recombinant interleukin-12 (IL-12) injected systemically inhibits transplantable mouse tumors (1-6) appears to rest on its induction of IFN- γ (2, 4), TNF- α (5) and GM-CSF (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), while their presence in the tumor microenvironment affects tumor cells directly by inducing the overexpression of MHC glycoproteins (13) and switching 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), since genetic screening is singling out not-yet patients with a defined genetic risk of cancer (18), and while pre-neoplastic 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 rat HER2/neu oncogene in the mammary gland. While 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 (20) (BALB-neuT) in their mammary glands were bred in our animal facilities (for details, see 16). A colony of FVB mice (N#202) carrying the rat HER2/neu protooncogene driven by the MMTV promoter (21) (FVB-neuN) was maintained under strict inbreeding from breeding pairs obtained from Dr. W.J. Muller, McMaster University, Hamilton, Ontario as previously described (16). Groups of individually tagged virgin females were used. Their mammary glands were inspected weekly and tumor masses measured with calipers in the two perpendicular diameters (16). Progressively growing masses >3mm mean diameter were regarded as tumors. Their growth was monitored weekly until all ten mammary glands displayed a palpable tumor, or one tumor exceeded an average diameter of 1.5 cm, when mice were sacrificed for humane reasons. Surviving BALB-neuT mice were sacrificed at the 33rd week when tumor masses were evident in all ten mammary glands, FVB-neuN mice at 90 weeks when they displayed a mean of 2.5 tumors/mouse.

IL-12 administration. 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. 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. Since 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 latter group are shown. The first course consisted of 50 ng IL-12/day, the subsequent six courses 100 ng IL-12/day. These seven courses were administered at different times (Fig. 1). BALB-neuT mice assigned to the "Chronic" treatment received the first course at the second week of age. From the 5th to the 25th week, courses were repeated every fourth week. Mice assigned to the "Late" treatment received the courses from the 13th to the 25th week. They were treated for two consecutive weeks followed by two weeks off. Mice of the "Early" treatment group received IL-12 administered beginning at the 2nd week and ending at week 14th. 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 fourth week starting on the 6th ("6-week-old" treatment), 22nd ("22-week-old" treatment) or 28th ("28-week-old" treatment) weeks of age. All 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, while similar groups of FVB-NeuN mice were sacrificed at weeks 15, 20, 22, 25, 27, and 30. For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, formalin-fixed, paraffin-embedded or acetone-fixed cryostat sections were incubated for 30 min with anti-endothelial cells (mEC-13.324) (22), and anti-proliferating cell nuclear antigen (PCNA) (Ylem, Rome, Italy) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat anti-rat, and mouse anti-goat IgG (Vector Labs., Burlingame, CA) for 30 min. Unbound antibodies were 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 three pathologists in a blind fashion. Two or more samples (one per tumor growth area) and ten 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 x 400 field (x 40 objective and x 10 ocular lens; 0.180 mm² per field). The rate of immunoreactivity for PCNA was obtained by counting the number of positive cell/number of total cells in the ductular and lobular structures under a microscope x 600 field (x 60 objective and 10 ocular lens; 0.120 mm² per field).

Statistical analysis. Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test, those in tumor/mouse numbers, number of microvessels and PCNA immunoreactive cells 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 and ref.16). Atypical hyperplasia of small lobular ducts and lobules is already evident at the second 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. 2a). At nearly 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 lower panel). Invasive lobular carcinomas (Fig. 4a) progressively develop and at the 33rd week tumor masses are palpable in all ten 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, they 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 of the number of mammary glands with a palpable tumor at 33 weeks when the experiment was ended were observed as compared to 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 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 (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 "Late" treatment group that had received two IL-12 courses only at that time. In the latter, however, distinct vascular damage associated with few reactive cells close to hyperplastic and neoplastic lobules was evident. Mice from "Chronic" and "Early" treatment groups revealed a less widely distributed atypical hyperplasia. Rare foci of carcinoma in situ were present in tissues from mice of the "Chronic", but not in those from the "Early" treatment groups (not shown). At week 25, invasive carcinomas were present the MSA controls (Fig. 4a). At this time the IL-12 regimens resulted in distinct pathological features. Either in situ or invasive carcinomas were evident in the mammary glands of mice from the "Chronic" and "Late" treatment groups (Fig. 4b, c). These lesions were smaller and less widely distributed than in MSA controls, and were even less pronounced in the "Chronic" treatment group. By contrast, a restrained atypical hyperplasia with foci of carcinoma in situ only was evident in mice from the "Early" treatment group (Fig. 4d).

Inhibition of tumor vasculature in BALB-neuT mice

This IL-12 induced delay of carcinogenesis closely fits the inhibition of tumor angiogenesis 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, while a few capillaries only surrounded foci of in situ carcinoma. Minor vascular damage and inhibition of angiogenesis were evident in mice from the "Late" treatment group. By contrast, a defective vascular network and a moderate and a marked reduction of the number of microvessels was evident in mice from the "Chronic" and "Early" treatment groups (Fig. 2a,b,c). These differences markedly diminished at the 25th week, when evident tumors were present in all treatment groups (Table 1, Fig. 2d,e,f).

Proliferative rate of BALB-neuT tumors

To evaluate whether IL-12 treatments affect the rate of growth of evident tumors, the time required by a tumor with a mean diameter of 4 mm to reach 8 mm mean diameters 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 all treatment groups. Evaluation of PCNA positive cells, too, failed to disclose appreciable differences within the treatments (Table 1).

Efficacy of lower IL-12 dosages in BALB-neuT mice

As IL-12 appears to effectively inhibit the progression of HER2/neu carcinogenesis, the dose range in which such inhibition is achieved was next 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 nor reduction of the number of mammary glands with a palpable tumor, but only a slight delay in tumor onset was found (Fig. 5).

Prevention of carcinogenesis in FVB-neuN mice

In FVB-NeuN mice the overexpressed *neu* protooncogene induces mammary carcinomas with a much longer latency time. Until the 22nd week, their mammary glands are histologically normal, while foci of atypical hyperplasia and carcinoma in situ become evident in a few glands of 25-week-old mice. Randomly a few of them slowly progress toward invasive carcinoma, and a mean of 2.5 tumors/mouse is evident at the 60th week. "6 week-old" and "22-week-old" IL-12 treatment 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 to MSA controls (Fig. 6). By contrast, "28-week-old" treatment was almost ineffective. It began when focal hyperplasia and carcinoma in situ are already a common finding.

Discussion

With distinct kinetics, transgenic female mice carrying the activated (BALB-neuT) or the protooncogene (FVB-neuN) rat HER2/neu under MMTV promoter progress towards 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. Present findings extend and confirm previous observations with mice treated with IL-12 during the whole progression of mammary carcinogenesis (16). Noguchi et al. have previously shown that a similar IL-12 treatment also inhibits chemical carcinogenesis in mice (15).

As 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 in which stage of tumor progression these 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 (18) and early diagnosis programs are detecting pre-neoplastic lesions (19).

As 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 evolution of the tumor:host angiogenic relationship, rather than intrinsic proliferative properties of transformed mammary cells is the point of no return for IL-12 activity. In effect, present findings suggest that at least part of this activity is due to IL-12's ability 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 the 13th-17th week, hyperplasia progresses to in situ carcinoma (16, and present findings). Immunohistochemically staining with anti-CD31 monoclonal antibody shows that rich microvascularisation inside preneoplastic lesions corresponds with their progression towards 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 second to the fourteen week induced both a scanty vascularization and poorly developed hyperplastic foci.

The importance of the time of IL-12 administration was further assessed with FVB-neuN mice, in whom an overexpressed *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 "22-week-old" treatment the first course was markedly delayed, it still started before an evident spreading of pre-neoplastic 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. 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 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 (5, 7). The anti-angiogenic (4, 8) and angiotoxic (26) activity of these two cytokines is stronger on those fragile capillary sprouts, which go with the shift from the pre-neoplastic to the neoplastic condition. Downstream mediators elicited by IL-12 may also act on neoplastic cells in which they downregulate the production of pro-angiogenic molecules (7, 27) and upregulate the release of anti-angiogenic factors as IP-10 and MIG (7, 14). Following 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. 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 (3, 7). With tumors that progress slowly, anti-angiogenic 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, since only patients bearing advanced tumors are enrolled in clinical trials (28).

The anti-tumor 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 blocking of tumor neoangiogenesis, important as this may well be. In effect, further subdivision of the "Early" protocol in shorter treatment periods markedly reduced IL-12 efficacy (not shown).

The lower efficacy of "Chronic" versus "Early" treatment could indicate that continuous IL-12 administration is suppressive (29), though 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 daily 100 ng/day IL-12 (i.e. around 4.5-7.7 μ g/Kg). This dose is well tolerated and almost no side effects were manifested (7, 16). It is probably close to the optimal active dose, since a ten or twenty-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 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 immunologic 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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Notes

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

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

*Performed on cryostat sections decorated with anti-endothelial (CD31) monoclonal antibody. At least 10 fields/sample were counted. Values expressed as mean ± SD of five 15- and 25-week-old mice.

#Performed on paraffin-embedded tissue sections with anti-PCNA monoclonal antibody

^sValues significantly different ($P > 0.001$) than in MSA controls.

Captions to figures

Fig. 1. Treatment outline. Black squares show the weeks in which mice received five-day courses (Monday through Friday) of daily intraperitoneal injections of IL-12 or MSA only during the progression of HER2/neu mammary carcinogenesis.

Fig. 2. Vascularization of the 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 (panel a). In hyperplastic mammary tissue from mice from the "Chronic" treatment group, a clear reduction of the number of microvessels is evident (panel b). Mice from the "Early" treatment group (panel c) display a marked reduction associated with a defective vascular network. At 25 weeks of age, the differences in vascular architecture of the neoplastic lesions from MSA control mice (panel d), "Chronic" (panel e) and "Early" (panel f) treatment groups are less evident.

Fig. 3. Progression of mammary carcinogenesis in BALB-neuT mice receiving "Chronic", "Late" or "Early" administration of IL-12. Percentage of tumor-free mice (upper panel) and mean number of palpable mammary carcinomas per mouse calculated as cumulative number of incident tumors/total number of mice (lower panel). Fifty

mice were the MSA controls. There were 40 in the "Chronic" treatment group, and 20 in both the "Early" and the "Late" treatment groups.

Statistical analysis in the upper panel shows that both "Early" and "Chronic" curves are significantly different ($p < 0.0005$ at least, Mantel Haenszel test) from the MSA curve, while the "Late" curve is not significantly different. After week 21 all values in the lower panel of "Early", "Chronic" and "Late" groups are significantly different from the corresponding values of the MSA group ($p < 0.05$ at least, by Student's t test).

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 (panel a). Multiple foci of carcinoma in situ associated with some hyperplastic islets were their main feature in mice from the "Chronic" treatment group (panel b), while both invasive carcinomas and large carcinoma in situ were present in mice from the "Late" treatment group (panel c). A restrained hyperplasia and few foci of carcinoma in situ are evident in mice from the "Early" treatment group (panel d).

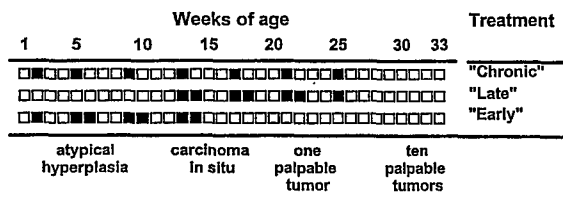
Fig. 5. Progression of mammary carcinogenesis in BALB-neuT mice from the "Early" treatment group that received daily intraperitoneal injections of 10 and 2 ng of IL-12. Percentage of tumor-free mice (upper panel) and mean number of palpable mammary carcinomas per mouse calculated as cumulative number of incident tumors/total number of mice (lower panel).

Statistical analysis: Each group consists of ten mice. In the upper panel both IL-12 curves were not significantly different from the MSA curve by the Mantel Haenszel test. Values of both 10 and 2 ng IL-12 groups 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).

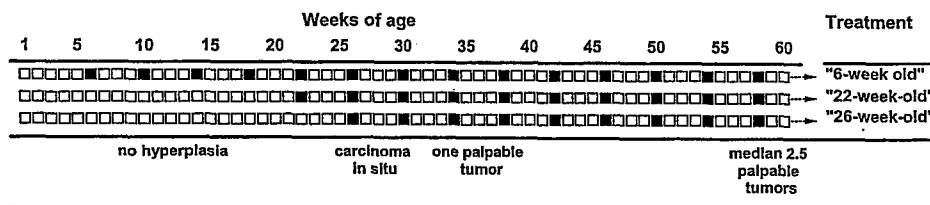
Fig. 6. Progression of mammary carcinogenesis in FVB-neuN mice receiving IL-12 treatment starting 6 ("6-week-old"), 22 (22-week-old) or 28 (28-week-old) week of age. Percentage of tumor-free mice (upper panel) and mean number of palpable mammary carcinomas per mouse calculated as cumulative number of incident tumors/total number of mice (lower panel). Twenty-six mice were in the MSA control group, twelve in the "28-week-old group", and eight in both the 6-week-old and the "22-week-old" groups.

Statistical analysis: in the upper panel both "6-week-old" and "22-week-old" curves were significantly different ($p < 0.025$ at least with Mantel Haenszel test) from the MSA curve, while the "28-week-old" curve was not statistically different from MSA. In the lower panel, after week 48 all values of both "6-week-old" and "22-week-old" were significantly different ($p < 0.05$ by Student's t test) from corresponding values of the MSA treatment. No value of the "28-week-old" curve was significantly different from the corresponding value of the MSA treatment.

BALB-neuT mice

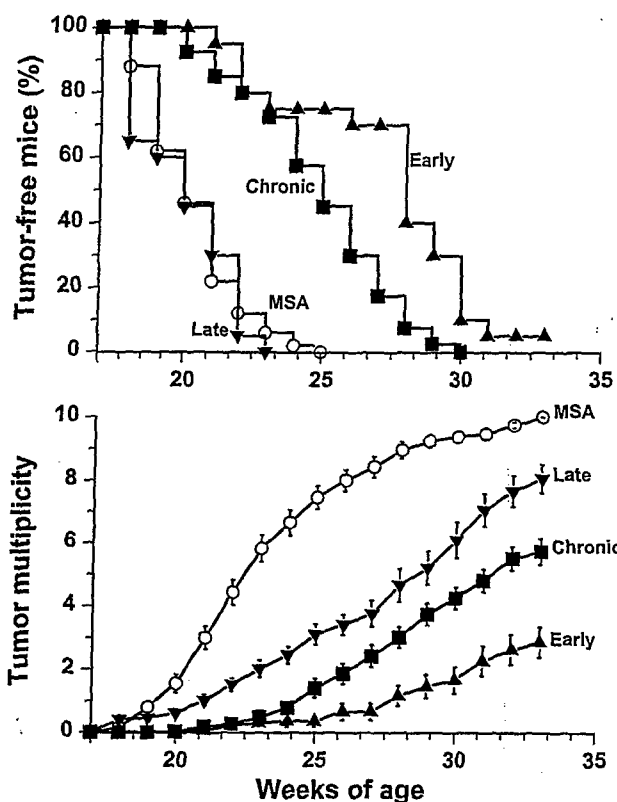


FVB-neuN mice

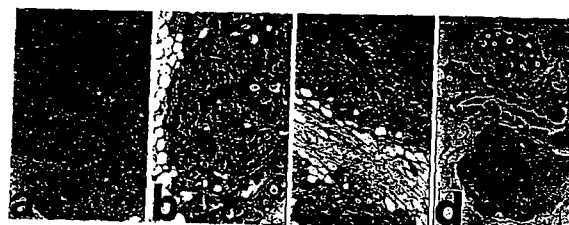


Boggio et al. Fig 1

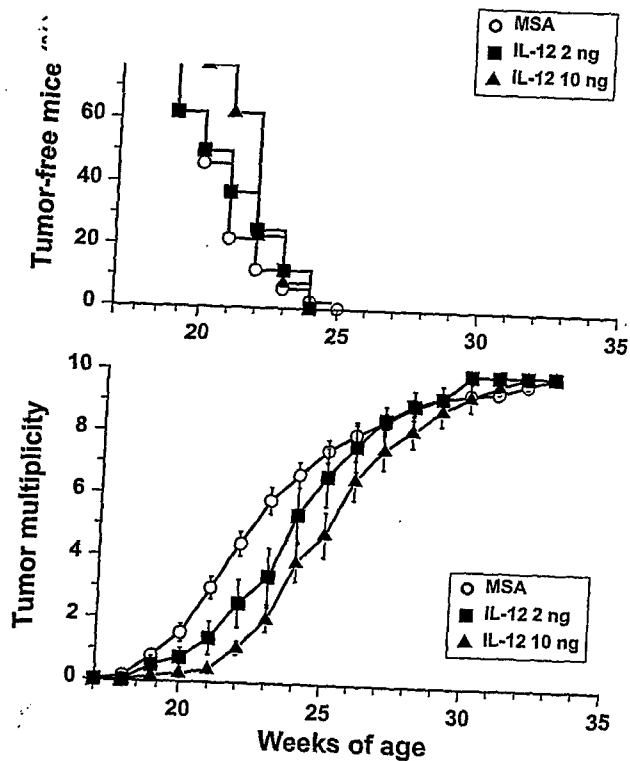
Boggio et al. Fig 2



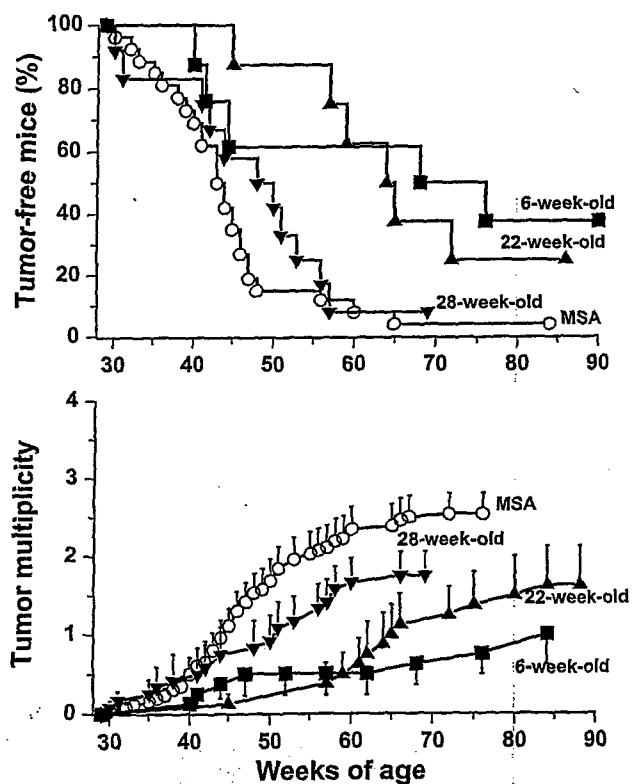
Boggio et al. Fig 3



Boggio et al Fig 4



Boggio et al Fig 5



Boggio et al. Fig 6

Appendix #5. Nanni et al., p185^{neu} protein is required for tumor and anchorage-independent growth, not for cell proliferation of transgenic mammary carcinoma,(to be submitted)

p185^{neu} protein is Required for Tumor and Anchorage-Independent Growth, not for Cell Proliferation of Transgenic Mammary Carcinoma¹

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Running title: p185^{neu} in transgenic mammary carcinoma

Key words: p185^{neu}, transgenic mice, mammary carcinoma, anchorage-independent cell growth, tumorigenicity

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³The abbreviations used are: DMEM, Dulbecco's modified minimal essential medium; FBS, fetal bovine serum; MMTV, mouse mammary tumor virus; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

ABSTRACT

Transgenic FVB-NeuN mice (N#202) bearing the rat *neu* proto-oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer develop focal mammary carcinoma expressing the *neu*-encoded p185^{neu} protein. Cell lines and clones derived from these spontaneous mammary tumors all contained the transgene, as confirmed by analysis of genomic DNA, but displayed heterogeneous levels of p185^{neu} protein phosphorylated in tyrosine residues (N202.1A and N202.1B) or no detectable p185^{neu} (N202.1E and TT3), as determined by FACS, Western blot and Northern blot analyses. Transgenic mice challenged with N202.1A and N202.1B cells developed fast-growing tumors with a short latency, while tumors in mice challenged with p185^{neu}-negative N202.1E and TT3 cells showed a very delayed onset and invariably overexpressed the p185^{neu} oncoprotein. The lower growth ability of p185^{neu}-negative cells *in vivo* was also confirmed in athymic nude mice. *In vitro* studies revealed a higher level of anchorage-dependent clonogenic growth and proliferation in N202.1E and TT3 cells as compared to N202.1A cells and 1E grown *in vivo* (1E-neu+) overexpressing p185^{neu} ($p < 0.05$), thus explaining the relative ease with which p185^{neu}-negative cell lines and clones were established *in vitro*. By contrast, analysis of anchorage-independent growth in soft agar revealed colony formation from p185^{neu}-positive but not p185^{neu}-negative cells ($p < 0.01$). 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 ($p < 0.001$). 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.

INTRODUCTION

The rat *neu* proto-oncogene and its human homologue *HER-2* encodes a 185 kDa transmembrane glycoprotein (p185^{HER-2/neu}) with intrinsic tyrosine kinase activity related to the epidermal growth factor receptor (1, 2). Amplification and overexpression of *HER-2* has been implicated in the pathogenesis of several human malignancies, including breast, ovarian and lung carcinomas (3-5), and its overexpression correlates significantly with poor prognosis in subsets of patients with breast cancer (6-8). *In vitro* studies using rodent cells indicated that p185^{neu} overexpression *per se* is sufficient to induce malignant transformation (9). In several strains of transgenic mice carrying the activated rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV)¹ 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 (10). FVB mice (line N#202) overexpressing the unactivated *neu* transgene under the transcriptional control of the MMTV promoter/enhancer in the mammary epithelium develop focal mammary tumors, but only in females and with long latency (11). 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 (12-16). Clinical and molecular data indicate a much lower incidence of *HER-2* gene amplifications in distant metastases than in primary tumors (17), 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 (18).

The role of *HER-2/neu* gene in tumor transformation and progression is still unclear. Some experimental data suggest that *HER2/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 (7). However, other data suggest a role for *HER2* gene in metastatic potential or in induction of hormone independence in a manner unrelated to cell proliferation (19, 20). Furthermore, the dual role of *HER2* activation in proliferation or differentiation depending on the cell type has been demonstrated either by transfection of the *HER2* gene or by treatment of cells with anti-p185^{HER-2} antibodies (21-23).

To investigate the role of *HER-2/neu* overexpression in tumorigenesis, we used the mouse strain N#202, which is transgenic for the unactivated rat *neu* gene (11) 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*.

MATERIALS AND METHODS

Transgenic mice and spontaneous mammary carcinomas. A colony of FVB-neuN mice, transgenic line N#202 (11, 24), was established in our animal facilities from breeding pairs obtained from Dr. William J. Muller, McMaster University, Hamilton, Ontario, Canada. 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 (25).

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% CO₂ 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.

Analysis of in vitro cell growth and proliferation. Growth on plastic was studied by seeding 5×10^3 viable cells in 25 cm² 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. For clonogenic growth on plastic, 200-6400 cells were seeded in 60-mm 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. Anchorage-independent growth in agar was determined by suspending $10^4 - 2 \times 10^5$ 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 (Oncogene Research Products, Cambridge, MA) or of an isotype-matched antibody of unrelated specificity.

Tumorigenicity and metastasis studies. Healthy young (8-16 weeks old) transgenic mice or 5-week-old *nu/nu* 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 s.c. 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 $\pi/6 \cdot [a \cdot b]^2$ 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.

Flow cytometry. The product of the transgene, rat *neu*, was detected using monoclonal antibody 7.16.4. Cells were stained in a standard indirect immunofluorescence procedure (26) with primary antibody followed by a fluorescein-conjugated anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, MD). After final washings, cells were resuspended in PBS containing 1 μ g/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.

Morphologic analysis. For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μ m, 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 Laboratories, Burlingame, CA) for 30 min. After washing to remove unbound Ig, slides were incubated with avidin biotin complex/alkaline phosphatases (Dako, Glostrup, Denmark).

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, 0.5% Triton X-100) containing protease plus phosphatase inhibitors, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM sodium orthovanadate (Na_2VO_4). Cell lysates were cleared by centrifugation at 4°C for 10 min at 10,000 x 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 μ g/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 μ l), and after 3 hr incubation, immune complexes were washed three times with lysis buffer, eluted and denatured 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 μ g/ml; Upstate Biotechnology, Inc., Lake Placid, NY) and rabbit polyclonal anti-p185^{neu} serum C-18 (2 μ g/ml; Santa Cruz Biotechnology, Inc.) followed by incubation with anti-mouse Ig and/or anti-rabbit Ig

horseradish peroxidase-linked whole antibodies (1:10000) (Amersham) and visualized using the ECL detection system (Amersham) according to the supplier's instructions.

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 MgCl₂, 0.01% gelatin, 0.45% Tween 20, 0.45% NP40, 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* proto-oncogene were: 5'-AGGGCAACTTGGAGCTTACCTACG-3' and 5'-GGGTTCTGCCTGGG-GTGGGA-3'.

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 & Schuell, Keene, NH) and immobilized by UV-crosslinking. Hybridization was carried out using a [³²P]dCTP (Amersham) probe, obtained by BamHI digestion of *neu* cDNA corresponding to 2248 bp of 3' end, using a random-primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). After stripping, the membrane was hybridized with a control [³²P]dCTP β-actin probe (Oncogene Research Products).

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 narrow distribution of p185^{neu} expression was observed within each tumor (Fig. 1), 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. 5 A,B).

Selection of p185^{neu}-negative cells by cloning. 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. 2, left).

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. 2, 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. 3 A). *neu* mRNA was not detected in p185^{neu}-negative cells (N202.1E and TT3) by Northern blot analysis (Fig. 3 B) or RT-PCR (data not shown), 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. 3 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. 3 D).

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. 4). Nevertheless, progressive tumors eventually appeared 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. 4).

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 (data not shown).

Lung metastases were detected in all mice i.v. injected 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. 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. 5F). 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. 5E with A and C).

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 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. 4).

p185^{neu} expression hampers anchorage-dependent growth but favors anchorage-independent 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-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. 7A), explaining the relative ease with which p185^{neu} loss variants emerged during *in vitro* culture. By contrast, N202.1A and 1E-neu+ cells formed large colonies in soft agar, while N202.1E produced no colonies at any cell concentrations tested, and TT3 cells formed only rare small clusters (Fig. 7A, Fig. 8). Moreover N202.1A colony

formation and growth in agar were significantly inhibited in the presence of an anti-p185^{neu} monoclonal antibody, but not of an irrelevant antibody (Fig. 7B).

Saturation density. The better adherent growth of p185^{neu}-negative cells resulted from a higher clonogenic growth on plastic surfaces and from higher proliferation rates, as revealed by bromodeoxyuridine labeling (data not shown). In contrast, growth of p185^{neu}-negative cells in confluent cultures was significantly less than that of p185^{neu}-positive cells which continued their growth and reached higher saturation densities (Fig. 7A).

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 negatively affect anchorage-dependent cell proliferation and growth. However 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}-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 (9, 27) 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 (28). In human prepartum and lactating mammary specimens, p185^{HER2} expression was detected at an intensity comparable to that of breast carcinomas (29). In another study, transfection of *HER-2* gene into MCF-7 breast carcinoma cells, appeared to increase differentiation of these cells (22).

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 (30). 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 (31). We suggest that changes in the level of p185^{HER-2/neu} expression might be causally related to variations in the dormant status of breast carcinoma.

In conclusion, our results indicate that p185^{HER-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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FIGURE LEGENDS

Fig. 1. Cytofluorometric analysis of p185^{neu} expression in enzymatically dissociated cells from mammary carcinomas of FVB-NeuN transgenic mice. A: median fluorescence intensity of p185^{neu} expression in 53 consecutive tumors. 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.

Fig. 2. Heterogeneous p185^{neu} expression in different in vitro 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.

Fig. 3. 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 lack of crossreactivity of rat *neu* primers with murine *neu* sequences. Controls: neg = no template; tail = positive control DNA extracted from tail of transgenic FVB-NeuN mice. GAPDH = glyceraldehyde-3-phosphate dehydrogenase. 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.

Fig. 4. 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.

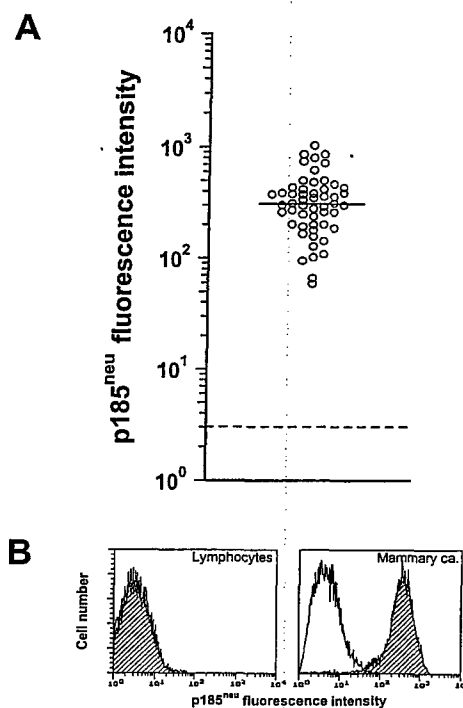
Fig. 5. Histological features (A, C, E) and immunohistochemical analysis for p185^{neu} expression (B, D, F) of mammary tumors grown in FVB-NeuN mice. A, B: spontaneous tumor; C, D: tumor induced by s.c. injection of N202.1A cells; E, F: long-latency tumor induced by s.c. injection of N202.1E cells. N202.1A and N202.1E cells both gave rise to a tumor morphologically similar to the spontaneous tumor. Anti-p185^{neu} immunostaining revealed a homogeneous pattern of distribution of p185^{neu} expression in all tumors. A, C, E: hematoxylin-eosin, magnification: 630x; B, D, F: immunohistochemistry, magnification: 630x.

Fig. 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.

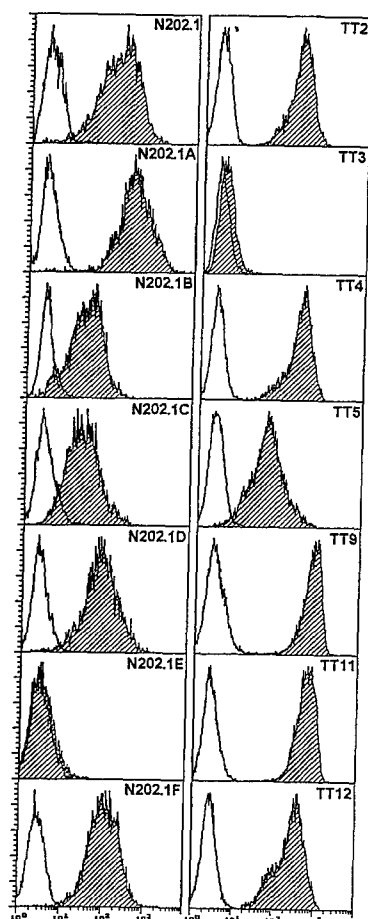
Fig. 7. A: Anchorage-dependent and -independent growth of transgenic mammary carcinoma cell lines in vitro. Data are the mean ($\pm$ SE) from at least 3 experiments. * = $P < 0.05$ and ** = $P < 0.01$ using Student's t test in comparison to p185^{neu}-positive cells. B: Inhibition of anchorage-independent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

Fig. 8. Anchorage-independent growth of transgenic mammary carcinoma cell lines positive or negative for p185^{neu} expression. Magnification: 25 $\times$.

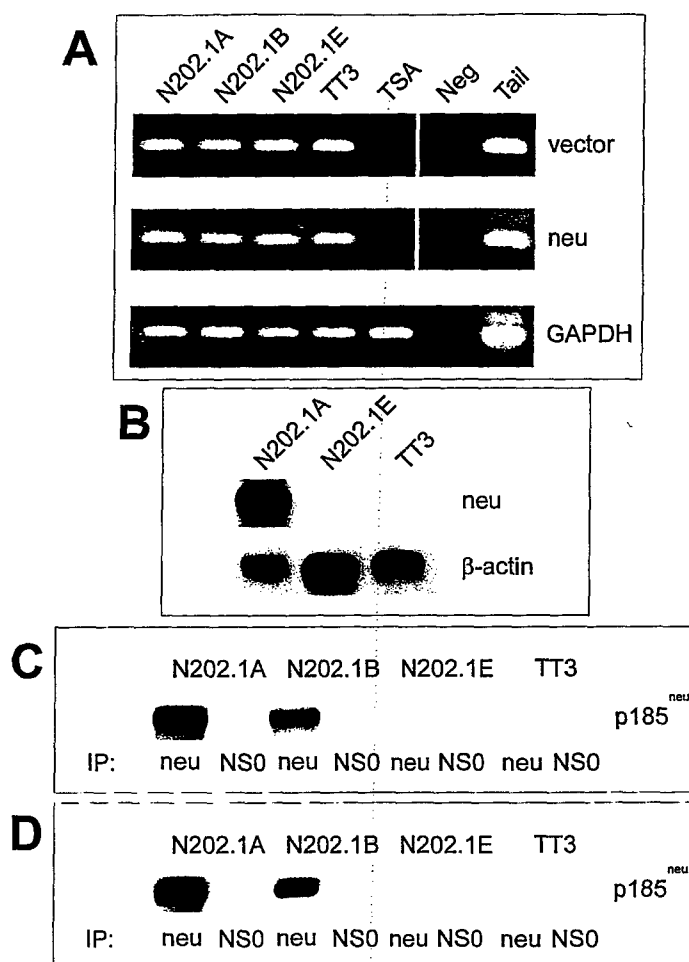
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Figure 1



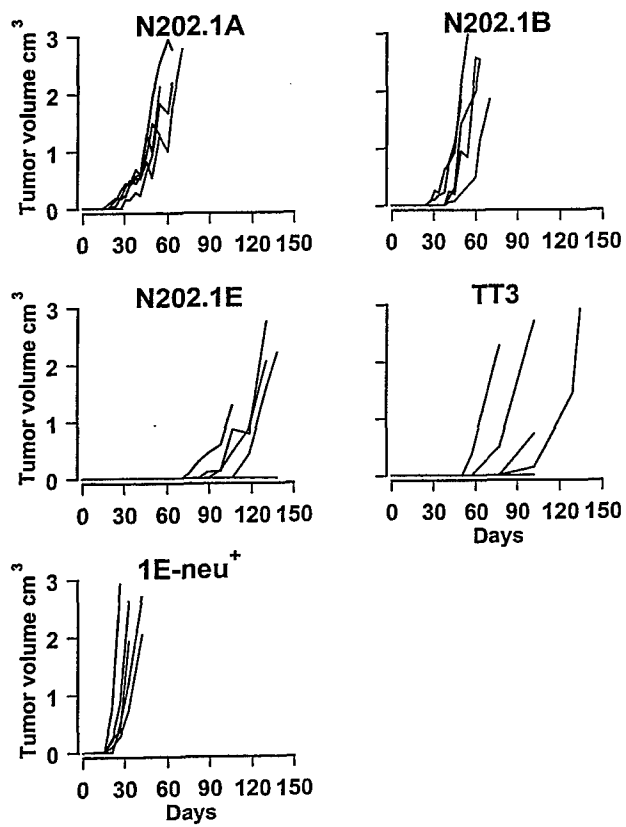
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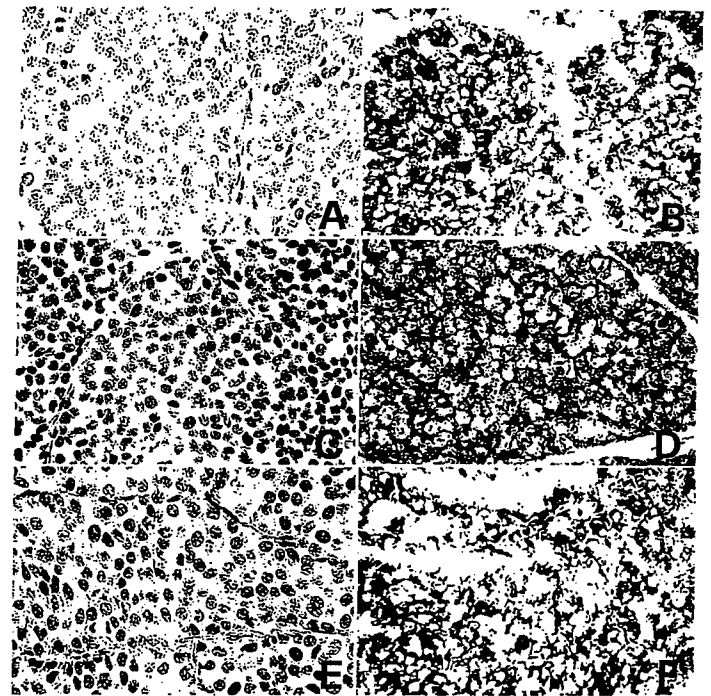
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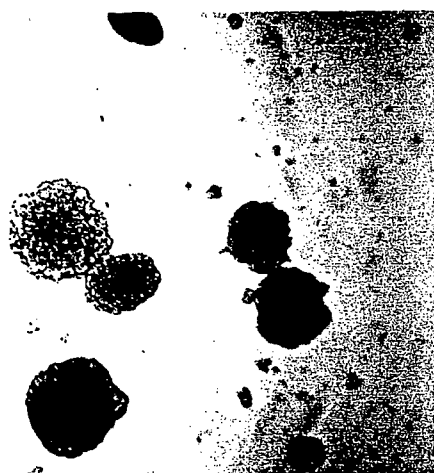
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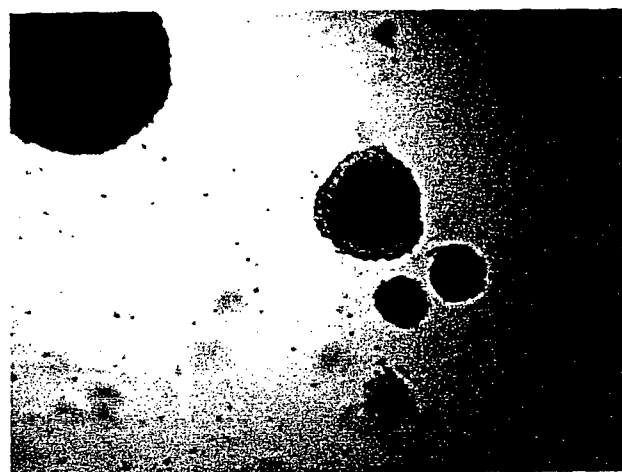
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Figure 8

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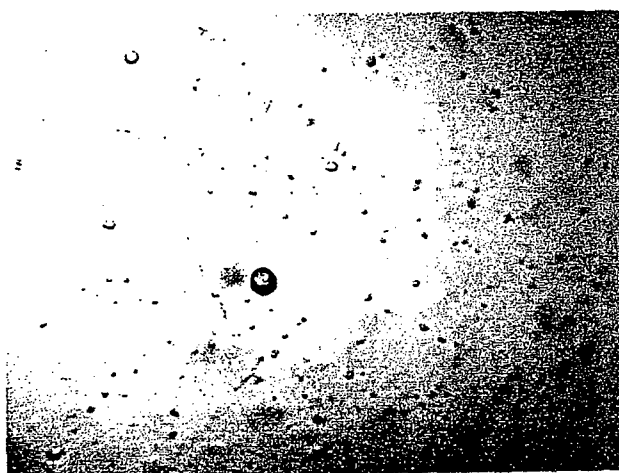
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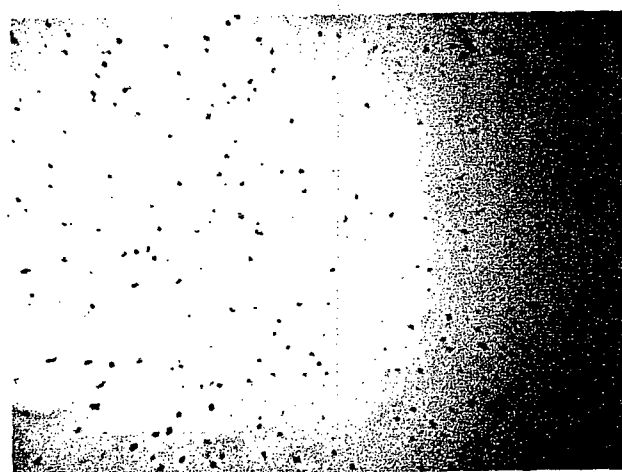
1E-neu⁺



TT3



N202.1E





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