Antimetastatic Activity of a Preventive Cancer Vaccine

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Abstract

The development of prophylactic cancer vaccines that protect healthy hosts from tumor development leaves open the question whether such vaccines are also effective against established tumors and metastases. We tested the therapeutic activity of a proven prophylactic anti-HER-2/neu vaccine against successive stages of mammary carcinoma progression in HER-2/neu transgenic mice. The vaccine consisted of transgenic mammary carcinoma cells expressing HER-2/neu and two adjuvants: allogeneic class I histocompatibility antigens and interleukin (IL)-12. Vaccination of mice bearing lung micrometastases resulted in a 90% inhibition of metastasis development, whereas vaccination of mice with incipient local tumors was ineffective. The antimetastatic response was hampered by immune tolerance, as the protection of transgenic mice against metastases was lower than that of wild-type congenics not tolerant to HER-2/neu. A significant gain in immunotherapeutic activity in transgenic mice was obtained through the coadministration of anti-CD25 monoclonal antibody targeting regulatory T cells, which resulted in a >99% inhibition of metastasis. The immune responses elicited in transgenic mice comprised the activation of lung granulocytes and macrophages and of systemic adaptive responses based on helper T cells and their cytokines (IFN-γ and IL-4) and anti-HER-2/neu antibodies. Dissection of relevant antimetastatic mechanisms by means of knockout mice and of depleting antibodies revealed a major difference between tumor prevention, which was completely dependent on anti-HER-2/neu antibodies, and metastasis therapy, which was antibody independent. In conclusion, a vaccine successfully developed for cancer immunoprevention showed a strong therapeutic activity against lung metastases mediated by protective immune mechanisms distinct from those preventing the onset of primary mammary carcinoma. [Cancer Res 2007;67(22):11037–44]

Introduction

Immunoprevention and immunotherapy are two main applications of immunology to oncology. Immunoprevention implements immunologic maneuvers, mainly vaccines, before tumor onset, to induce an immune response that protects the host from carcinogenesis and tumor progression. Immunotherapy consists of immunologic interventions administered to cancer patients to eliminate existing tumor cells.

Various cancer immunoprevention studies have stressed the differences between the principles, applications, and immune mechanisms of immunoprevention and immunotherapy (1–4). For example, it was repeatedly shown that the long-term efficacy of vaccines used to prevent carcinogenesis is mediated more by antibodies than by those cytotoxic T cells that are the mainstay of classic cancer immunotherapy (5, 6).

We argued that it is unlikely that preclinical immunoprevention results will be directly conducive to prevention studies in humans (1, 7). A series of phase I and II studies in cancer patients is likely to be the first human implementation of vaccines designed for immunoprevention (7). From this state of facts, two important questions arise. (a) Are immunopreventive vaccines endowed with therapeutic activity? If this is not the case, then the design and feasibility of early clinical studies will meet formidable obstacles. (b) If instead effective immunopreventive vaccines also have therapeutic activity, are the latter mediated by the same immune mechanism that prevent carcinogenesis?

To answer these questions, we studied the therapeutic activity of a cell vaccine against HER-2/neu, which was previously shown to be very active in the prevention of mammary carcinogenesis in HER-2/neu transgenic mice (8, 9), mainly through the induction of HER-2/neu–specific antibodies (5). We show here that the same vaccine has a considerable therapeutic activity against metastatic mammary carcinoma, mediated by immune responses partially distinct from those at work in long-term immunoprevention of carcinogenesis.

Materials and Methods

Mice. BALB/cAnNCrlBR (BALB/c) mice were purchased from Charles River Italy. BALB-neuT mice (H-2d haplotype) overexpressing the activated rat HER-2/neu oncogene driven by the mouse mammary tumor virus promoter, IFN-γ gene knockout BALB/c mice, and μMT mice (knockout for the immunoglobulin μ chain gene) were bred, maintained, and genetically screened as reported (5, 10). Rag2−/−γc−/− breeders were kindly given by the Central Institute for Experimental Animals (Kawasaki, Japan); mice were then bred in our animal facilities under sterile conditions. Experiments were authorized by the institutional review board of the University of Bologna and done according to Italian and European guidelines. Individually tagged virgin female mice of 6 to 9 weeks of age were used for the experiments.

Cells. TUBO cellclone (referred to as Neu/H-2d) was derived from a mammary carcinoma of BALB-neuT mouse (11), and TT12 cell clone was derived from a mammary carcinoma of FVB-neuN #202 (H-2b haplotype origin, transgenic for the rat neu proto-oncogene). TT12 cells express high levels of p185 neu and are referred to as Neu/H-2b (12). Transfection of the IL-12 genes in Neu/H-2b cells, giving rise to the Neu/H-2b/IL-12 transfectant, was described previously (9). Cells were cultured in DMEM supplemented with 20% fetal bovine serum (FBS; Life Technologies) at 37°C in a humidified 5% CO2 atmosphere.

Vaccination protocol against tumor onset. The vaccination protocol consisted of 4-week cycles: in the first 2 weeks, mice received four
twice-weekly i.p. vaccinations with $2 \times 10^6$ mitomycin C–treated (40 μg/mL) cells in 0.4 mL PBS followed by 2 weeks of rest. For the chronic protocol vaccination, cycles started at the 6th week of age and were repeated lifelong, and in the early, late, and very late protocols, mice received only the first three vaccination cycles starting at 6, 10, and 16 weeks of age, respectively; vaccinations against incipient mammary carcinomas started as soon as mice had one palpable tumor mass and continued until sacrifice.

**Lung metastases and therapeutic vaccination.** For the induction of lung micrometastases, mice received i.v. 2.5 × 10⁴ Neu/H-2d cells. Mice were sacrificed 33 days after cell injection and subjected to an accurate necropsy. Lungs were stained with black India ink to better outline metastases and fixed in Fekete’s solution. Lung metastases were counted using a dissection microscope. Therapeutic vaccination started 1 or 7 days after metastasis induction. Schedule included two weekly administrations of cell vaccines (prepared as described above) repeated until sacrifice. Control groups included untreated mice and mice treated twice weekly with 100 ng/mouse of recombinant IL-12 (kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA).

**In vivo cell depletions.** All depletion studies were done according to previously standardized protocols (13). T-cell depletion studies were done by i.p. injection of anti-CD8 (2.43), anti-CD4 (GK1.5), or anti-CD25 (PC61) rat monoclonal antibodies (mAb), all from the American Type Culture Collection.

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**Figure 1.** Inhibition of mammary carcinogenesis in BALB-neuT female mice subjected to various vaccination protocols with Neu/H-2d/IL-12 cells. **A,** tumor-free survival curves of groups of six to seven mice. Each arrow represents one monthly cycle of vaccinations administered for the entire lifetime of the mouse starting at 6 wk of age (chronic protocol) or for 3 mo starting at different mouse ages. The early protocol started at 6 wk of age, the late protocol at 10 wk, and the very late protocol at 16 wk. Statistical significance: early and late protocols, $P < 0.01$ versus untreated mice; very late protocol, not significantly different from untreated mice. A modified version of A was previously published in a review article (7). CIS, carcinoma in situ. **B,** tumor multiplicity, same mice as in A. Each curve shows the cumulative number of mammary carcinomas per mouse. **C,** vaccination of BALB-neuT mice bearing palpable mammary carcinomas. Columns, mean interval in days between the day of tumor onset and the day in which the mouse (10 per group) had to be sacrificed because of tumor burden; bars, SE. No vaccination group was statistically different from untreated mice by the Student’s $t$ test.
Collection. All mAbs were first administered i.p. 24 h after micrometastasis induction (i.e., 6 h before the first vaccination). Treatment with anti-CD4 and anti-CD8 mAbs was then repeated 24 h after the first vaccination and 6 h before the second, third, fourth, sixth, and eighth vaccination. The anti-CD25 treatment was repeated 6 h before the fifth vaccination. The mAb dosages were 200 μg/mouse (anti-CD8) or 500 μg/mouse (anti-CD4 and anti-CD25). For natural killer (NK) depletion, mice received i.v. 0.4 mL of a 1:30 dilution of anti-asialo GM1 antiserum (Wako) 48 h after micrometastasis induction (24 h before the first vaccination); this protocol was found to avoid well-known effects of NK depletion on metastatic seeding.

Immune responses. Mixed lymphocyte-tumor cell cultures (MLTC) were done with spleen cells cocultured at a 50:1 ratio with proliferation-blocked restimulator cells for 6 days in RPMI 1640 supplemented with 10% FBS and with 20 units/mL of recombinant interleukin (IL)-2 as reported previously (9). The cytotoxic ability of lymphoblasts was tested in secondary cultures against target cells by a standard 51Cr-release assay, and the percentage of lysis was calculated as described (14). Supernatants from MLTC primary cultures done as above with a 10:1 splenocytes to tumor cell ratio were assayed for IFN-γ and IL-4 by ELISA assays (9). Sera collected from individual mice were used as primary

Figure 2. Metastatic development and antimetastatic immune responses induced by vaccination. A, mammary carcinoma metastases developing in the lungs of BALB-neuT mice 7 d after the i.v. injection of 2.5 × 10⁵ Neu/H-2d cells. Top, H&E. Magnification, ×400. Bottom, HER-2/neu immunohistochemistry. Magnification, ×400. B, HER-2/neu-stained metastases in the lungs of untreated and vaccinated mice. Magnification, ×25. Vaccination started 7 d after metastasis induction, and mice were sacrificed 21 d later. C, infiltration of lung metastases by macrophages, granulocytes, and CD4⁺ T cells in mice vaccinated 7 d after i.v. tumoral cell injection. Immunohistological stainings done on control and vaccinated mice showed in vaccinated mice an increase in the presence of CD4⁺ T cells (magnification, ×200) 7 d after vaccination and of macrophages and granulocytes 14 d after vaccination (magnification, ×630). D, electron micrograph showing a metastatic aggregate occupying the lumen of a lung capillary lined by endothelial cells (E) and flat protrusions of pneumocyte cytoplasmas. A macrophage (M) is in close contact with the epithelial tumoral cell (T). A severely damaged cell with clear aspects of necrosis (N) is visible at the periphery of the aggregate. Magnification, ×2,800.
Results

Loss of vaccine efficacy versus progressive tumor stages. Prophylactic, lifelong vaccination of cancer-prone HER-2/neu transgenic mice with cells expressing HER-2, allogeneic MHC antigens, and IL-12 (triplex vaccine, “chronic” protocol, Fig. 1A) completely prevented the onset of mammary carcinoma (9). To compare the prophylactic and therapeutic potential of the triplex vaccine, we administered three monthly vaccination cycles to HER-2/neu transgenic mice starting at different ages, corresponding to atypical hyperplasia (“early” protocol), carcinoma in situ (“late” protocol), and incipient invasive carcinoma (“very late” protocol) as determined by previous pathologic studies (17). We found a progressive loss of vaccine efficacy directly related to the advancement of tumor progression, both in terms of tumor incidence (Fig. 1A) and tumor multiplicity (Fig. 1B). In particular, tumor onset was considerably delayed in mice receiving the early protocol compared with untreated mice, whereas the very late therapeutic protocol produced only a negligible delay. To evaluate the vaccine also against a quantifiable tumor burden, we treated mice with incipient mammary carcinomas (Fig. 1C). The triplex vaccine had little, if any, efficacy against this larger tumor target. Therefore, the triplex vaccine was extremely effective at preventing mammary carcinoma onset in tumor-free mice but was ineffective against established local tumors.

Therapy of lung metastases with the triplex vaccine. After local growth, the key step of malignant tumor progression is metastatic dissemination, which represents a restriction point akin to early tumor onset (18). HER-2/neu transgenic mice bearing advanced lung micrometastases produced by the i.v. injection of syngeneic Neu/H-2d mammary carcinoma cells received multiple administrations of the vaccine to evaluate its efficacy in blocking metastatic development. We purposely chose experimental conditions conducive to a high metastatic burden to obtain a realistic and stringent model of therapy. The 7-day delay between the i.v. challenge and the beginning of therapy is equal to one fourth of the

Table 1. Therapeutic vaccination of mice bearing lung micrometastases

<table>
<thead>
<tr>
<th>Mice</th>
<th>Start* of vaccination</th>
<th>Vaccination</th>
<th>Incidence</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-2/neu transgenic</td>
<td>Day 7</td>
<td>None</td>
<td>9/9 (100%)</td>
<td>&gt;200</td>
<td>134 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>IL-12</td>
<td>5/5 (100%)</td>
<td>86</td>
<td>42 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>Neu/H-2d</td>
<td>5/5 (100%)</td>
<td>117</td>
<td>89 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2e</td>
<td>4/4 (100%)</td>
<td>124</td>
<td>59 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d/IL-12</td>
<td>12/12 (100%)</td>
<td>26†</td>
<td>1–165</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>None</td>
<td>10/10 (100%)</td>
<td>&gt;200</td>
<td>80 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>IL-12</td>
<td>5/5 (100%)</td>
<td>60</td>
<td>23–153</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d</td>
<td>5/5 (100%)</td>
<td>81†</td>
<td>76 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d/IL-12</td>
<td>9/10 (90%)</td>
<td>3†</td>
<td>0–27</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Day 7</td>
<td>None</td>
<td>10/10 (100%)</td>
<td>&gt;200</td>
<td>47 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d</td>
<td>5/5 (100%)</td>
<td>79†</td>
<td>27–97</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2e</td>
<td>4/5 (80%)</td>
<td>11†</td>
<td>0–21</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d/IL-12</td>
<td>1/11 (9%)</td>
<td>0†</td>
<td>0–4</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>None</td>
<td>18/18 (100%)</td>
<td>&gt;200</td>
<td>95 to &gt;200</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d</td>
<td>0/5 (0%)</td>
<td>0†</td>
<td>0–0</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Neu/H-2d/IL-12</td>
<td>0/15 (0%)</td>
<td>0†</td>
<td>0–0</td>
</tr>
</tbody>
</table>

NOTE: Brackets indicate statistically significant comparisons by the Wilcoxon rank sum test.

*Days after the i.v. challenge with TUBO cells.
† P < 0.05 at least.
‡ P < 0.01 at least.
§ P < 0.001.

antibody to stain different target cells (and then subjected to cytofluorometric analysis) as described previously (8).

Morphologic analysis. Groups of three mice were killed at 7, 14, 21, and 30 days after cell injection. To optimize the detection of microscopic metastases and to ensure systematic uniform and random sampling, lungs were cut transversally to the trachea into 2.0-mm-thick parallel slabs with a random position of the first cut in the first 2.0 mm of the lung, resulting in five to eight slabs per lung. The slabs were then embedded cut surface down. Tissue samples were processed as described previously (15) for histologic evaluation. For immunohistochemistry, pyridoxal phosphate–fixed tissues were embedded in OCT and acetone-fixed cryostat sections were incubated for 60 min with the following: anti-c-ErbB2/HER-2; anti-proliferating cell nuclear antigen (clone PC10; DakoCytomation); anti-CD4 (Chemicon International); anti-Gr1, a cell surface protein mainly expressed by granulocytes (clone RB68C5); anti-CD45R/B220, a subset of mouse CD45 isoforms predominantly expressed on B lymphocytes; anti-CD11b mainly expressed on monocytes-macrophages (BD PharMingen); anti-Foxp3, nuclear transcription factor specific for regulatory T cells (Treg; clone MF333F, Alexis Italia); and a mix of antiendothelial cell (CD31, Chemicon International; CD31, clone MEC13.3, and CD105/endoglin, clone MJ7/18, BD PharMingen) antibodies. After washing, sections were overlaid with biotinylated goat anti-rat and anti-rabbit Ig (Vector Laboratories) for 30 min. Unbound Ig was removed by washing, and slides were incubated with avidin-biotin complex/alkaline phosphatase (DakoCytomation). For immunofluorescence to detect p185 expression, Alexa Fluor 488–conjugated secondary antibodies were used. For electron microscopy, lung lobes were fixed for 4 h in 2.5% glutaraldehyde in 0.2 mol/L HEPES buffer (pH 7.4) and processed as reported (16).


Cancer Res 2007; 67: (22). November 15, 2007 11040 www.aacrjournals.org Downloaded from cancerres.aacrjournals.org on January 27, 2018. © 2007 American Association for Cancer Research.
natural history of metastatic development in this system, which can be translated to some months of metastatic development in humans. Figure 2A shows the appearance and HER-2/neu expression of such 7-day metastatic lesions.

The triplex vaccine produced a >87% reduction in the number of lung metastases (Table 1). Treatment of mice with syngeneic (Neu/H-2\(d\)) or allogeneic (Neu/H-2\(q\)) cells expressing p185 or with recombinant IL-12 alone did not inhibit metastatic development (Table 1), thus illustrating the need for the simultaneous presence of the three immune stimuli encoded by the triplex vaccine (8).

The triplex vaccine had a significant antimetastatic activity; however, all vaccinated mice still had a sizable metastatic burden. We then explored three possible limiting factors of vaccine efficacy: (a) advancement of the metastatic stage, (b) immune tolerance of HER-2/neu gene product p185, and (c) Tregs.

**Therapy of early lung metastases.** Vaccination of transgenic mice bearing early (1 day) lung lesions (Table 1) produced a strong improvement in the inhibition of metastatic development, which increased to 99%. Significant inhibitions were also observed in mice receiving either allogeneic p185\(^+\) cells or IL-12 (Table 1). The simultaneous release of IL-4, which was already evident in unstimulated splenocyte cultures and increased significantly after restimulation with tumor cells (Fig. 3, middle). The simultaneous release of IL-4 (Fig. 3, bottom) indicates that the Th response was not completely polarized toward type I.

CTL assays against syngeneic mammary carcinoma target cells expressing HER-2/neu did not reveal any significant activity above background (data not shown). This agrees with the results of in vivo CD8 depletion shown below and with previous CTL studies with the same vaccine (8) because CD8 recognizing dominant p185 peptides with high affinity is lacking in transgenic mice due to central tolerance (25).

The study of semiserial sections showed that the number and the dimensions of lung metastases after 14, 21, and 28 days were lower in vaccinated mice than in control mice; moreover, the structure of metastatic lesions was frequently cribriform and less compact than in controls (Fig. 2B).

Immunohistochemical studies, done to analyze local antimetastatic immune responses in the lungs of vaccinated mice, revealed a scarce reactive infiltrate at all the time points examined.

### Table 2. Therapeutic vaccination of HER-2/neu transgenic mice selectively depleted of immune cell populations

<table>
<thead>
<tr>
<th>Depletion</th>
<th>Vaccination</th>
<th>Start(^a) of vaccinations</th>
<th>Incidence</th>
<th>Lung nodules</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>Day 7</td>
<td>12/12 (100%)</td>
<td>190</td>
</tr>
<tr>
<td>None</td>
<td>Neu/H-2(d)/IL-12</td>
<td>Day 7</td>
<td>5/5 (100%)</td>
<td>30</td>
</tr>
<tr>
<td>Treg</td>
<td>Neu/H-2(d)/IL-12</td>
<td>Day 7</td>
<td>4/6 (67%)</td>
<td>3</td>
</tr>
<tr>
<td>None</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 1</td>
<td>9/10 (90%)</td>
<td>3</td>
</tr>
<tr>
<td>Treg</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 1</td>
<td>2/6 (33%)</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 1</td>
<td>4/5 (80%)</td>
<td>4</td>
</tr>
<tr>
<td>CD4</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 1</td>
<td>6/6 (100%)</td>
<td>39</td>
</tr>
<tr>
<td>CD8</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 1</td>
<td>5/5 (100%)</td>
<td>11</td>
</tr>
<tr>
<td>None</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 3</td>
<td>5/5 (100%)</td>
<td>36</td>
</tr>
<tr>
<td>NK</td>
<td>Neu/H-2(q)/IL-12</td>
<td>Day 3</td>
<td>5/5 (100%)</td>
<td>41</td>
</tr>
</tbody>
</table>

**NOTE:** Brackets indicate statistical comparisons by the Wilcoxon rank sum test.

**Abbreviation:** ns, not significant.

\(^a\)Days after the i.v. challenge with TUBO cells.

\(^1\) \(P < 0.05\) at least.

\(^2\) \(P < 0.01\) at least.
IFN-γ Student's < 0.005, Student's 5 to 10 mice bled after seven vaccine administrations. Statistical significance:
glycoproteins (Neuneg/H-2q), p185neu (Neu/H-2d), or both (Neu/H-2q).
alone or with mitomycin C–treated tumor cells expressing allogeneic MHC by ELISA in the supernatants of spleen cells that were cultured t
by splenocytes of untreated and vaccinated mice. IFN-γ release of IL-4 by splenocytes of untreated and vaccinated mice, culture t
conditions as indicated above for IFN-γ. Statistical significance: P < 0.005, Student’s t test, Treg-depleted mice versus nondepleted mice.
restimulation with Neu/H-2q versus restimulation with Neu/H-2d.
release of this cytokine was elicited by vaccination (Fig. 3). To approach this issue, we vaccinated mice deficient in various immune responses bearing 7-day metastases.
A complete loss of vaccine efficacy in double-knockout Rag2<sup>−/−</sup> / γc<sup>−−</sup> mice draws the attention to the main cell types deficient in these mice (i.e., B, T, and NK cells and their products, such as antibodies and cytokines; Table 3).
Metastatic growth in antibody-deficient μMT mice was inhibited by vaccination (Table 3). We have previously shown that the triplex vaccine was totally ineffective in the prevention of tumor growth in mice lacking anti-HER-2 antibodies (5). Therefore, this result establishes an important difference between effector immune mechanisms mediating the activity of therapeutic or prophylactic vaccinations. The former seems now B-cell independent, whereas the latter was previously found to be completely dependent on antibody activity (5).

To analyze the contribution of T cells, we vaccinated CD4- and CD8-depleted HER-2/neu transgenic mice bearing lung micrometastases. CD8 depletion did not significantly modify the efficacy of vaccination (Table 2), thus suggesting that these cells were not required for metastasis therapy. This is in accordance with the negative results of in vitro CTL assays cited above. The significant, albeit not complete, loss of therapeutic efficacy observed in CD4-depleted mice (Table 2) indicates that T helper cells have an important role in the antimetastatic immune mechanisms elicited by the vaccine. NK cells did not play a major role in mediating the activity of the vaccine, as the antimetastatic activity of the vaccine was not affected by NK cell depletion (Table 2).

We previously found that the major contribution of cell-mediated immunity to cancer immunoprevention was through the release of cytokines, particularly IFN-γ (5, 8). In fact, a copious release of this cytokine was elicited by vaccination (Fig. 3). To ascertain whether IFN-γ played a causal role in the antimetastatic efficacy, we vaccinated IFN-γ−deficient mice (Table 3). In the absence of this cytokine, a significant loss of efficacy of the vaccine was found, and all mice developed a sizable metastatic burden, thus confirming the key role of IFN-γ.

Some experiments reported above concern immune mechanisms downstream of IFN-γ [e.g., NK activity (NK-depleted mice) and Ig class switch (μMT antibody-deficient mice)]. Moreover, the study of direct effects of IFN-γ on neoplastic cells showed that exposure of metastatic Neu/H-2d cells to IFN-γ inhibited their proliferation, up-modulated MHC class I expression, down-modulated the expression of growth- and malignancy-related molecules, such as HER-2/neu and matrix metalloproteinase-9, and strongly

Figure 3. Humoral and cellular immune responses induced by the triplex vaccine in BALB-neuT mice. Top, binding of sera (diluted 1:65) to Neu/H-2<sup>d</sup> vaccine cells as evaluated by cytofluorimetric analysis. Columns, mean of 5 to 10 mice bled after seven vaccine administrations. Statistical significance: P < 0.005, Student’s t test, vaccinated mice versus untreated; P < 0.05, Student’s t test, Treg-depleted mice versus nondepleted mice. Middle, release of IFN-γ by splenocytes of untreated and vaccinated mice. IFN-γ was assayed by ELISA in the supernatants of spleen cells that were cultured in vitro for 6 d alone or with mitomycin C–treated tumor cells expressing allogeneic MHC glycoproteins (Neu<sup>neu</sup>H-2<sup>d</sup>), p185<sup>Neu</sup>H-2<sup>α</sup> or both (Neu/H-2<sup>α</sup>). Columns, mean of 10 mice. Statistical significance: P < 0.001 at least, Student’s t test, all vaccinated groups versus nonvaccinated controls; P < 0.001, Student’s t test, restimulation with Neu/H-2<sup>d</sup> versus unstimulated or other restimulations. Bottom, release of IL-4 by splenocytes of untreated and vaccinated mice, culture conditions as indicated above for IFN-γ. Statistical significance: P < 0.01 at least, Student’s t test, all vaccinated groups versus nonvaccinated controls; P < 0.01, Student’s t test, restimulation with Neu/H-2<sup>d</sup> versus restimulation with Neu/H-2<sup>α</sup>.

However, a slight increase in the presence of CD4+ T cells at the 14th day of metastasis growth (Fig. 2C) and of macrophages and granulocytes at later time points was observed in vaccinated mice, whereas no difference was found in the presence of CD8+ T and B220+ B cells at any time point (data not shown). Foxp3+ Tregs were not observed in the lung of both vaccinated and control mice. Vascularization around and inside metastases and the expression of HER-2/neu were similar in vaccinated and control mice.

Electron microscopy showed that several macrophages in vaccinated mice were in close contact with tumoral cells, particularly at the metastasis periphery, where some of them showed aspects of necrosis (Fig. 2D).

**Antimetastatic immune mechanisms.** The complex immune response elicited by the triplex vaccine does not allow a dissection of relevant immune mechanisms, which effectively abrogated metastatic expansion, from bystander responses devoid of therapeutic activity. To approach this issue, we vaccinated mice deficient in various immune responses bearing 7-day metastases.

A complete loss of vaccine efficacy in double-knockout Rag2<sup>−/−</sup> / γc<sup>−−</sup> mice draws the attention to the main cell types deficient in these mice (i.e., B, T, and NK cells and their products, such as antibodies and cytokines; Table 3).

Metastatic growth in antibody-deficient μMT mice was inhibited by vaccination (Table 3). We have previously shown that the triplex vaccine was totally ineffective in the prevention of tumor growth in mice lacking anti-HER-2 antibodies (5). Therefore, this result establishes an important difference between effector immune mechanisms mediating the activity of therapeutic or prophylactic vaccinations. The former seems now B-cell independent, whereas the latter was previously found to be completely dependent on antibody activity (5).

To analyze the contribution of T cells, we vaccinated CD4- and CD8-depleted HER-2/neu transgenic mice bearing lung micrometastases. CD8 depletion did not significantly modify the efficacy of vaccination (Table 2), thus suggesting that these cells were not required for metastasis therapy. This is in accordance with the negative results of in vitro CTL assays cited above. The significant, albeit not complete, loss of therapeutic efficacy observed in CD4-depleted mice (Table 2) indicates that T helper cells have an important role in the antimetastatic immune mechanisms elicited by the vaccine. NK cells did not play a major role in mediating the activity of the vaccine, as the antimetastatic activity of the vaccine was not affected by NK cell depletion (Table 2).

We previously found that the major contribution of cell-mediated immunity to cancer immunoprevention was through the release of cytokines, particularly IFN-γ (5, 8). In fact, a copious release of this cytokine was elicited by vaccination (Fig. 3). To ascertain whether IFN-γ played a causal role in the antimetastatic efficacy, we vaccinated IFN-γ−deficient mice (Table 3). In the absence of this cytokine, a significant loss of efficacy of the vaccine was found, and all mice developed a sizable metastatic burden, thus confirming the key role of IFN-γ.

Some experiments reported above concern immune mechanisms downstream of IFN-γ [e.g., NK activity (NK-depleted mice) and Ig class switch (μMT antibody-deficient mice)]. Moreover, the study of direct effects of IFN-γ on neoplastic cells showed that exposure of metastatic Neu/H-2d cells to IFN-γ inhibited their proliferation, up-modulated MHC class I expression, down-modulated the expression of growth- and malignancy-related molecules, such as HER-2/neu and matrix metalloproteinase-9, and strongly
Discussion

The major conclusions that can be drawn from the results shown here are that a vaccine originally developed for cancer immunoprevention has a powerful therapeutic activity against micrometastases and that the immune mechanisms that mediate this therapeutic activity are different from those involved in long-term protection from tumor onset.

With regard to tumor progression, the efficacy of the triplex vaccine displayed a distinct bimodal trend, with two maxima corresponding to the early development of primary and of metastatic neoplastic lesions, and a dramatic loss of efficacy against established local tumors. This establishes an important principle indicating that the vaccines developed for cancer immunoprevention are endowed with therapeutic activity, provided that they are deployed against appropriate targets, such as the early phases of metastasis development.

It is well known that cancer vaccines are poorly effective against advanced lesions; however, this piece of immunologic wisdom contains an implicit ambiguity. In fact, “advanced” could pertain either to the advancement of cancer progression from preneoplasia to metastasis or to establishment of large neoplastic lesions in symbiosis with the surrounding microenvironment. Our results clearly show that in the present model system the advancement of tumor progression does not entail resistance to immunotherapy because early lung metastases were as sensitive as early primary carcinomas. On the contrary, established local tumors were resistant to immune responses elicited by vaccines. Under this respect, it is important to stress that in our case the target antigen p185 fulfills the definition of “oncoantigen” (1), that is, it is a persistent antigen not easily lost during tumor progression because neoplastic growth in vivo strictly depends on its continuing expression, whereas antigen loss variants are no longer tumorigenic (12).

Immune tolerance of transgenic mice to HER-2/neu is a major obstacle to successful cancer immunotherapy and immunoprevention (19, 25–28), as illustrated here by the lower efficacy of the triplex vaccine in HER-2/neu–tolerant transgenic mice than in nontolerant, nontransgenic mice. In most instances, some antigen-specific lymphocyte clones are not deleted by negative selection and can be stimulated by appropriate immunologic maneuvers in the periphery of adult hosts (25). In our case, the triplex vaccine, combining the target antigen with two potent adjuvants such as IL-12 and allogeneic MHC class I, was powerful enough to halt the continuing carcinogenic process in the mammary glands of HER-2/neu transgenic mice but not enough to completely eradicate metastatic development. Depletion/inactivation of Tregs significantly boosted antimetastatic therapy and produced in tolerant transgenic mice an immune control of the metastatic process similar to that obtained with vaccine alone in nontolerant mice.

A comparison of the immune mechanisms mediating the cancer-preventive effects of the triplex vaccine with those responsible for its antimetastatic therapeutic efficacy revealed major differences. This was an unexpected outcome, given that the same vaccine and similar vaccination protocols were used both for prevention and for therapy.

Cancer prevention was previously found to be completely dependent on anti-HER-2/neu antibodies of the IgG2a and IgG2b subclasses, in turn dependent on appropriate immunoglobulin class switch mediated by IFN-γ (5). IFN-γ was the major mediator in common with metastasis therapy, whereas antibodies seemed devoid of significant therapeutic activity. Additional vaccine-induced mechanisms playing a causal role against metastases were CD4 cells (at the systemic level) and granulocytes and macrophages infiltrating tumor cell nests in the lungs.

In summary, long-term prevention of mammary carcinogenesis was mediated mainly by Th1 and humoral immunity, whereas rapid elimination of incipient lung metastases resulted from a combination of cytokine- and cell-mediated mechanisms, including inflammatory cells. A copious antibody response was induced by the vaccine in all instances; hence, it was astonishing to find that elimination of metastatic deposits could be achieved even in antibody-deficient mice. For what concerns the humoral immune response, the major differences between cancer prevention and metastasis therapy are the anatomic localization of neoplastic lesions and growth kinetics. It is likely that part of the effective antimitastatic responses, particularly those mediated by

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NOTE: Brackets indicate statistical comparisons by the Fisher’s exact test or the Wilcoxon rank sum test as appropriate.
Abbreviation: ns, not significant.
*<i>P < 0.01</i> at least.
macrophages and granulocytes, could be the result of lung localization, where local inflammatory responses play a major role in the defense of the host from foreign microorganisms (29). The growth kinetics of lung metastases induced by the i.v. injection of mammary carcinoma cells was clearly faster than that of spontaneous primary carcinomas. Hence, a likely explanation for the lack of relevance of the antibody response in metastasis therapy is that the time required for the vaccine to elicit a strong secondary IgG response was of the same order of magnitude as that of metastasis growth, whereas in the case of cancer prevention a full-fledged antibody response could reach its upper plateau when fully neoplastic cells are not yet present in the mammary gland (5, 17).

The fact that antimetastatic immunity elicited by the triple vaccine was independent of antibodies also hints that the spectrum of relevant IFN-\(\gamma\) actions in the present system was different from that involved in cancer immunoprevention. The pleiotropic activities of IFN-\(\gamma\) (30) include direct antitumor effects (anti-proliferative activity), indirect effects mediated by secondary chemokines (antiangiogenic activity of MIG and IP-10), and its broad range of activities as an internal regulator of host immune responses (Ig class switch, macrophage activation, Th cell differentiation, etc.). In cancer immunoprevention, we showed that the most relevant activity of IFN-\(\gamma\) was the induction of protective antibody responses because vaccine activity completely disappeared both in IFN-\(\gamma\) knockout mice, which no longer produced protective Ig isotypes, and in antibody-deficient mice, which, however, retained the capacity to produce IFN-\(\gamma\) (5). In the experiments shown here, mice lacking anti-HER-2/\(\alpha\) antibodies were protected from metastatic development, thus showing that in the therapeutic setup other activities of IFN-\(\gamma\) played a more fundamental role than Ig class switch.

We have shown here that an effective prophylactic vaccine is also therapeutically effective in a model of aggressive metastatic development. As clinical deployment of cancer-preventive vaccines faces many practical hurdles (7), this result opens up the possibility of early clinical testing in a therapeutic, rather than prophylactic, human context.

Acknowledgments

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This article is dedicated to the fond memory of Giorgio Prodi, 20 years after his untimely death.

References

Correction: Antimetastatic Activity of a Preventive Cancer Vaccine

In the article on antimetastatic activity of a preventive cancer vaccine in the November 15, 2007 issue of Cancer Research (1), there was an error in the printing of Table 3. The corrected Table appears below.

Table 3. Therapeutic vaccination of immunodeficient mice

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*P < 0.01 at least.
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