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

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ABSTRACT

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Mice. BALB/c mice overexpressing the activated rat HER2/neu oncogene driven by the mouse mammary tumor virus (MMTV) promoter (Ref. 20; BALB-neuT) in their mammary glands were bred in our animal facilities (for details, see Ref. 16). A colony of FVB mice (N9202) carrying the rat HER2/neu proto-oncogene driven by the MMTV promoter (Ref. 21; FVB-neuN) was maintained under strict inbreeding from breeding pairs obtained from Dr. W. J. Muller (McMaster University, Hamilton, Ontario, Canada) as described previously (16). Groups of individually tagged virgin females were used. Their mammary glands were inspected weekly, and tumor masses were measured with calipers in two perpendicular diameters (16). Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored weekly until all 10 mammary glands displayed a palpable tumor or until one tumor exceeded an average diameter of 1.5 cm, at which time mice were sacrificed for humane reasons. Surviving BALB-neuT mice were sacrificed at the 33rd week, when tumor masses were evident in all 10 mammary glands; FVB-neuN mice were sacrificed at 90 weeks, when they displayed a mean number of 2.5 tumors/mouse.

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

Histological and Immunohistochemical Analysis. Groups of three IL-12-treated and untreated BALB-neuT mice were killed at 15, 25, and 30 weeks of age, whereas similar groups of FVB-neuN mice were sacrificed at weeks 15, 20, 22, 25, 27, and 30. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. The stages of carcinogenesis were determined according to the criteria of Muller et al. (16). For the study of immunohistochemical parameters, the sections were treated with 0.01% hydrogen peroxide to block endogenous peroxidase activity. The following monoclonal antibodies were used: anti-CD3 (clone 4B12, DAKO), anti-CD4 (clone RM-5, DAKO), anti-CD8 (clone 53-6.7.7, DAKO), anti-IL-12 (clone 12-3 (6B5), Genetics Institute), anti-IFN-γ (clone 1A9, Oncogene Science), and anti-Ki-67 (clone 6F2, Novocastra). The sections were stained with the biotin-peroxidase method (Vector Laboratories). The slides were counterstained with hematoxylin. The number of cells per field was counted on images captured with a digital camera and analyzed using a software package for image analysis (AxioVision, Carl Zeiss, Jena, Germany). The number of Ki-67-positive cells and the number of CD3-positive cells were counted in 10 high-power fields per section and reported as the mean ±SD.

Received 8/11/99; accepted 11/12/99.

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1 Supported by the Italian Association for Cancer Research, the Istituto Superiore di Sanità, Special project gene therapy, CNR Target project on Biotechnology, University of Bologna (fund for selected research topics), Ministero dell’Università e della Ricerca Scientifica, and by the Department of the Army, USA. Grant DAMD17-98-1-8030 (to G. F.). “The information contained in this paper does not necessarily reflect the position or the policy of the U.S. government, and no official endorsement should be inferred.

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3 The abbreviations used are: IL, interleukin; MSA, mouse serum albumin; MMTV, mouse mammary tumor virus; PCNA, proliferating cell nuclear antigen.
and stained with H&E or Giemsa. For immunohistochemistry, formalin-fixed, paraffin-embedded, or acetone-fixed cryostat sections were incubated for 30 min with antiendothelial cells (mEC-13.324; Ref. 22) and PCNA (Ylem, Rome, Italy) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat antirat and mouse antigoat IgG (Vector Laboratories, Burlingame, CA) for 30 min. Unbound antibodies were removed by washing, and the slides were incubated with avidin-biotin complex/alkaline phosphatase (DAKO, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blind fashion. Two or more samples (one per tumor growth area) and 10 randomly chosen fields in each sample from mice with multiple hyperplastic foci or tumors were evaluated for each determination. Individual microvessels were counted under a microscope (×400 field (×40 objective and ×10 ocular lens; 0.180 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 ×600 field (×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; differences in tumor/mouse numbers, the number of microvessels, and PCNA immunoreactive cells were evaluated by Student’s t test.

RESULTS

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

To evaluate the ability of IL-12 to inhibit this progression, mice received seven 5-day courses of IL-12 at different times (Fig. 1). In the chronic treatment, the courses started in the 2nd week and continued until the 25th week. Both a delay in the onset of the first mammary tumor and a 50% reduction in the number of mammary glands with a palpable tumor at 33 weeks (when the experiment was ended) were observed as compared with MSA controls (Fig. 3). To assess whether IL-12 is also effective during later phases, other mice were first treated at the 13th week of age, when hyperplasia takes the form of a carcinoma in situ. Courses continued until the 25th week. This late treatment did not delay the onset of the first tumor but did reduced the number of tumors at week 33 by 22%. The early treatment began at the 2nd week and continued until week 14. The delay in onset of the first tumor and the reduction in the number of tumors are significantly higher than those seen in the chronic treatment group. When the early treatment was further split into shorter 4-week administration schedules, much less protection was observed (data not shown).

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

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**BALB-neuT mice**

<table>
<thead>
<tr>
<th>Weeks of age</th>
<th>Treatment</th>
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<tr>
<td>1</td>
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<td>5</td>
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<tr>
<td>30</td>
<td></td>
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<tr>
<td>33</td>
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</table>

- atypical hyperplasia
- carcinoma in situ
- one palpable tumor
- ten palpable tumors

**FVB-neuN mice**

<table>
<thead>
<tr>
<th>Weeks of age</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>&quot;6-week old&quot;</td>
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- no hyperplasia
- carcinoma in situ
- one palpable tumor
- median 2.5 palpable tumors

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Fig. 1. Treatment outline. ■, weeks in which mice received 5-day courses (Monday through Friday) of daily i.p. injections of IL-12 or MSA only during the progression of HER2/neu mammary carcinogenesis.
atypical hyperplasia. Rare foci of carcinoma in situ were present in tissues from mice of the chronic treatment group, but not in those from the early treatment group (data not shown). At week 25, invasive carcinomas were present in the MSA controls (Fig. 4a). At this time, the IL-12 regimens resulted in distinct pathological features. Either in situ carcinomas or invasive carcinomas were evident in the mammary glands of mice from the chronic and late treatment groups (Fig. 4b and c). These lesions were smaller and less widely distributed than those in MSA controls and were even less pronounced in the chronic treatment group. In contrast, a restrained atypical hyperplasia with foci of carcinoma in situ only was evident in mice from the early treatment group (Fig. 4d).

**Inhibition of Tumor Vasculature in BALB-neuT Mice.** This IL-12-induced delay of carcinogenesis closely fits the inhibition of tumor angiogenesis as assessed by direct microvessel count (Table 1). At 15 weeks, mammary glands from the MSA controls displayed vigorous capillary sprouts inside the atypical hyperplastic areas, whereas only a few capillaries surrounded the foci of carcinoma (Fig. 2a). Mice from the chronic treatment group (c) display a marked reduction associated with a defective vascular network. At 25 weeks of age, the differences in the vascular architecture of the neoplastic lesions from the MSA control mice (d), chronic (e), and early (f) treatment groups are less evident.

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

**Efficacy of Lower IL-12 Doses in BALB-neuT Mice.** Because IL-12 appears to effectively inhibit the progression of HER2/neu carcinogenesis, the dose range in which such an inhibition is achieved was evaluated. When early treatment was performed using 10 and 50 times lower doses of IL-12, no delay in the appearance of the first tumor or reduction of the number of mammary glands with a palpable tumor was found, but a slight delay in tumor onset was seen (Fig. 5).
Fig. 4. Histopathology of mammary lesions in 25-week-old BALB-neuT mice. Invasive carcinomas formed by a uniform population of round cells grouped in alveolar structures are evident in the mammary glands of MSA controls (a). Multiple foci of carcinoma in situ associated with some hyperplastic islets were the main feature in mice from the chronic treatment group (b), whereas both invasive carcinomas and large carcinoma in situ were present in mice from the late treatment group (c). A restrained hyperplasia and a few foci of carcinoma in situ are evident in mice from the early treatment group (d).

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

<table>
<thead>
<tr>
<th>IL-12 treatment</th>
<th>MSA only</th>
<th>Chronic treatment</th>
<th>Late treatment</th>
<th>Early treatment</th>
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<tr>
<td>Microvessel count&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>15th week</td>
<td>22 ± 3</td>
<td>13 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19 ± 3</td>
<td>9 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>25th week</td>
<td>13 ± 2</td>
<td>12 ± 2</td>
<td>13 ± 4</td>
<td>11 ± 2</td>
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<tr>
<td>% of PCNA immunoreactivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30th week&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23 ± 6</td>
<td>21 ± 5</td>
<td>27 ± 9</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Doubling time of the diameter (4–8 mm) of the first mammary tumor</td>
<td>8 ± 5</td>
<td>12 ± 8</td>
<td>25 ± 19</td>
<td>25 ± 18</td>
</tr>
</tbody>
</table>

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

<sup>b</sup> Values are significantly different (P < 0.001) from those of MSA controls.

<sup>c</sup> Performed on paraffin-embedded tissue sections with anti-PCNA monoclonal antibody.

Prevention of Carcinogenesis in FVB-neuN Mice. In FVB-neuN mice, the overexpressed neu proto-oncogene induces mammary carcinomas with a much longer latency time. Until the 22nd week, the mammary glands of these mice are histologically normal, whereas foci of atypical hyperplasia and carcinoma in situ become evident in a few glands of 25-week-old mice. Randomly, a few of them progress slowly toward invasive carcinoma, and a mean number of 2.5 tumors/mouse is evident at the 60th week. The 6-week-old and 22-week-old IL-12 treatments began when FVB-neuN mice were still free from macroscopic or microscopic mammary lesions (17). Both treatments significantly reduced tumor incidence and multiplicity as compared with MSA controls (Fig. 5). In contrast, 28-week-old treatment was almost ineffective. It began when focal hyperplasia and carcinoma in situ were already a common finding.

DISCUSSION

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

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

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

Around the 2nd week, almost all mammary glands of BALB-neuT mice display multiple foci of ductular atypical hyperplasia. Between...
atypical hyperplasia and then toward carcinoma. The 28-week-old protocol confers only a negligible protection. During the 28th week appears to be of critical importance because the carcinomas and their multiplication. The period between the 22nd and 26th week is assessed with FVB-neuN mice, in whom an overexpressed neu oncogene induces mammary carcinomas after a markedly longer latency on July 20, 2017. © 2000 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from

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

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

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

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

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

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

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

We thank Prof. John Iliffe for editing the manuscript.

REFERENCES


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