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Cooperative Effects of *Mycobacterium tuberculosis Ag38* Gene Transduction and Interleukin 12 in Vaccination against Spontaneous Tumor Development in Proto-*neu* Transgenic Mice[1]

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ABSTRACT

An approach to stimulating an immune response against tumors is to transduce tumor cells with bacterial genes, which represent a "danger signal" and can induce a wide immune response. *Mycobacterium tuberculosis* genes and their encoded proteins play a pivotal role in linking innate and cell-mediated adaptive immunity and represent ideal candidates as immune adjuvants for tumor vaccines. The efficacy of a cancer vaccine, obtained by transduction of a mammary tumor cell line with the *M. tuberculosis Ag38* gene, was investigated in female mice transgenically expressing the rat *HER-2/neu* proto-oncogene. These mice spontaneously develop stochastic mammary tumors after a long latency period. The onset of spontaneous mammary tumors was significantly delayed in mice vaccinated with *Ag38*-transduced cells but not in mice vaccinated with nontransduced cells as compared with untreated mice. Protection from spontaneous tumor development was increased when mice were vaccinated with the mycobacterium gene-transduced vaccine plus a systemic administration of interleukin 12 (IL-12) at a low dose. Mice vaccinated with nontransduced cells plus IL-12 developed tumors, with only a slight delay in tumor appearance as compared with the control group. Lymphocytes obtained from lymph nodes of mice vaccinated with transduced cells secreted high levels of IFN-γ. $CD3^+CD8^+$ spleen cells derived from these mice responded to the tumor with IFN-γ production. These data indicate the efficacy of a short-term protocol of vaccinations exploiting the adjuvant potency of a *M. tuberculosis* gene and low doses of IL-12 in a model of stochastic development of mammary tumors. This adjuvant approach may represent a promising immunotherapeutic strategy for cancer immunization.

INTRODUCTION

Most investigations in the field of cancer gene therapy have been related to the antitumor effect of cancer vaccines with transfected cytokine genes, major histocompatibility antigens, and costimulatory molecules (1–3). Another approach to stimulating an immune response against tumors is to transduce tumor cells with bacterial genes, which represent a "danger signal" and can induce a wide immune response. Indeed, in less than a decade, the archetypal view that the immune system exists primarily to distinguish "self" from "non-self" has been replaced by the paradigm that the immune system functions primarily to distinguish dangerous from nondangerous antigens (4). Presumably, the immune system has evolved over millions of years to respond to bacteria with a rapid activation of defenses that are best suited to fight microbial infection. *Mycobacterium tuberculosis* is a major target in this fight, and studies on complete Freund's adjuvant indicate that the mycobacterium contains a number of substances that stimulate the immune response and promote Th1 differentiation (5). In a study to identify the fraction of bacillus Calmette-Guérin responsible for its antitumor activity, Tokunaga *et al.* (6) implicated the DNA component. Subsequent studies demonstrated that bacterial DNA containing CpG motifs enables the vertebrate innate immune system to sense "danger" via pattern recognition receptors with broad reactivity (7). In the light of the pivotal role of *M. tuberculosis* genes and their encoded proteins in linking innate and cell-mediated adaptive immunity, these bacterial substances are promising candidates to be used as adjuvants for the development of effective therapeutic or prophylactic tumor vaccines. The immune response they elicit might facilitate the activation of the immune system against tumor antigens and the eventual selective destruction of tumor cells through a specific immune response. Indeed, studies in different countries have shown that neonatal bacillus Calmette-Guérin vaccination confers some degree of protection against leukemia and other childhood cancers (8).

We observed previously significant protection against tumor development in mice immunized with the melanoma cells transduced with a *M. tuberculosis* gene encoding the M_r 38,000 protein (*Ag38*), one of the most immunogenic antigens of this bacterium (9, 10). However, like nearly all cancer vaccines, only a slight therapeutic effect was observed in mice with existing tumors.[3] The limited efficacy in curing existing tumors is thought to rest primarily in inadequate penetration of the tumor mass by the immune cells and in the escape of some tumor cell progeny from the immune response.

The recent discovery of gene mutations that predispose to cancer now enables identification of at-risk individuals with a defined genetic prognosis (11). The goal of vaccination in such individuals is the recruitment of the immune system in eliminating single transformed cells before tumor nodules develop.

In this study we evaluated the efficacy of a cancer vaccine in proto-*neu* transgenic mice. In this strain, the expression of proto-*neu* induces the development of spontaneous focal mammary tumors in all females (12), although the stochastic development of the tumors and the long latency period indicate the requirement for additional events in tumor formation. The vaccine was obtained by transduction of a mammary tumor cell line, derived from a transgenic mouse tumor, with the *M. tuberculosis Ag38* gene. We also tested whether systemic coadministration of low-dose rIL-12[4] might enhance vaccine efficacy. Delayed onset and decreased incidence of tumor development were observed with vaccine plus rIL-12 as the most effective protocol.

MATERIALS AND METHODS

Cell Lines. Tumor cell lines N202.1A, N202.1E, and TT3, each derived from a mammary carcinoma spontaneously grown in FVB-neuN transgenic

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[3] Unpublished results.

[4] The abbreviations used are: rIL-12, recombinant murine IL-12; RT-PCR, reverse transcription-PCR; mAb, monoclonal antibody; PE, phycoerythrin; SRB, sulforhodamine B; Th1, T helper 1; Th2, T helper 2.

mice (12), were obtained from Dr. P. Nanni (Istituto di Cancerologia, Bologna, Italy). *Ag38*-transduced N202.1A cells were obtained and selected as described (9). Transduced cells were maintained in G418 selection medium (0.5 mg/ml) and analyzed for bacterial product expression by RT-PCR using specific oligonucleotides and by FACScan analysis with HBT12 mAb.

Mice and *in Vivo* Experiments. FVB-NeuN transgenic mice (12), on the H-2q FVB inbred background and carrying the rat *HER-2/neu* proto-oncogene under the transcriptional control of the mouse mammary tumor virus promoter, were bred and treated in accordance with institutional guidelines. In one set of experiments, virgin females were vaccinated s.c. twice at a 4-week interval with 10^6 irradiated (20,000 rads) transduced or nontransduced cells or left untreated. Mammary glands were inspected weekly, and two perpendicular diameters of tumor masses were recorded. In the second experiments, mice received injections i.p. with rIL-12 (kindly provided by Dr. L. Adorini, Roche Milano Ricerche, Milan, Italy) immediately after vaccination with irradiated cells and on days +1, +2, and +3 (150 ng/day diluted in PBS containing 100 μg/ml mouse serum albumin). Differences between groups were analyzed using the log-rank test.

Cytokine Production by Lymph Node Cells. Mice were vaccinated s.c. into the right hind footpad with 5×10^6 nontransduced or transduced irradiated cells alone or in the presence of rIL-12 injected i.p. (150 ng/day on days 0, +1, +2, and +3). Six days later, mice were sacrificed, and popliteal lymph nodes were aseptically removed and pooled from two mice in each group. Lymphocytes were mechanically dissociated and cultured (2×10^5 cells/well) in 96-well flat-bottomed plates precoated with anti-CD3 mAb (1 μg/well) at 37°C for 18 h. Supernatant was collected and tested for IFN-γ and IL-4 production by ELISA kits (EuroClone Ltd., Devon, United Kingdom; Genzyme, Cambridge, MA). Two-tailed unpaired *t* test was used to evaluate differences between groups.

To evaluate the role of IL-12 on cytokine production, mice were treated i.p. for 4 days with 1 mg of purified rat anti-murine IL-12 p40 antibody (clone 17.8 IgG2a; kindly provided by Dr. G. Trinchieri, The Wistar Institute of Anatomy and Biology, Philadelphia, PA; Ref. 13) or with the same dose of an unrelated antibody. Six h after the first treatment with the antibody, mice were vaccinated s.c. into the right hind footpad with 5×10^6 transduced cells alone or with 5×10^6 nontransduced cells in the presence of rIL-12, injected i.p. (150 ng/day for 4 days). Experiments were then carried on as described above.

Tumor-stimulated Splenocyte Culture and FACScan Analysis. Splenocytes obtained from mice 7 days after vaccination with 10^6 irradiated transduced cells, irradiated nontransduced cells plus IL-12 (150 ng/day on day 0, +1, +2, and +3), or irradiated nontransduced cells alone were cultured and restimulated weekly at a density of 5×10^5/well in 24-well plates with irradiated (20,000 rads) N202.1A tumor cells (5×10^4) and IL-2 (25 units/ml). Syngeneic irradiated (3000 rads) splenocytes (1×10^6) were added to each well as feeder cells.

For FACScan analysis of cells responding to the tumor, lymphocytes were harvested and cultured for 21 h with immobilized anti-CD3 mAb and 1 μM monensin added for the last 12 h (Sigma, Milan, Italy). Cells were suspended in PBS, fixed with 4% paraformaldehyde for 5 min at room temperature, and permeabilized with PBS-saponin (0.2%) for 15 min at room temperature. Cells were triple-stained with Cy-Chrome-labeled anti-CD8 or anti-CD4, FITC-anti-IFN-γ, and PE-anti-IL-4 mAbs. Single staining was performed with FITC-anti-CD3 mAb. Isotype-matched FITC- or PE- or Cy-Chrome-conjugated mAbs were used for background determination. All mAbs were purchased from PharMingen and diluted as indicated in the supplier's sheet. Cytofluorometric analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA), and 50,000 events were acquired.

To evaluate IFN-γ production in response to *neu*-expressing or non-expressing tumor cell lines, 2×10^5 lymphocytes/well were stimulated with 10^5 irradiated (20,000 rads) N202.1A or N202.1E cells, respectively, or cultured alone in 96-well plates in the presence of IL-2 (25 units/ml). After 24 h, supernatant were recovered, and IFN-γ levels were determined by ELISA as above.

Cell Proliferation Assay. Inhibition of N202.1A, N202.1E, and TT3 tumor cell growth by IFN-γ was determined using an SRB assay. SRB stains for cellular proteins. Briefly, cells were seeded at 1.5×10^3 cells/well in 96-well microplates in 200 μl of culture medium alone or in the presence of various concentrations of murine IFN-γ (0.1, 1, 10, 100, and 1000 ng/ml; Peprotech, London, United Kingdom). After removing culture medium, cells were fixed in 50% trichloroacetic acid at 4°C for 1 h, washed five times with distilled water, and stained with 1% acetic acid-0.4% (w/v) SRB solution at room temperature; after 30 min, plates were washed five times with 1% acetic acid and air-dried. SRB bound to cellular proteins was dissolved by addition of 10 mM Tris-HCl (pH 10.5) to each well. Absorbance at 550 nm, proportional to the number of cells attached to the culture plate, was measured by spectrophotometry. Each test was performed in quadruplicate.

RESULTS

To obtain endogenous expression of the *M. tuberculosis* protein encoded by the *Ag38* gene, N202.1A tumor cells, derived from a mammary carcinoma spontaneously grown in a proto-*neu* transgenic mouse, was transduced *in vitro* with the retroviral vector pLAg38TMSN, carrying the *M. tuberculosis Ag38* gene (9). Selected cell clones produced the *M. tuberculosis Ag38* transcript as detected by RT-PCR (Fig. 1*a*) and expressed the bacterial protein on the cell surface, as detected by the anti-Ag38 protein mAb HBT12 on FACScan analysis (Fig. 1*b*).

The protection induced by the cancer vaccine against spontaneous tumor development was evaluated in proto-*neu* transgenic virgin female mice. Mice were randomly divided into three groups and vaccinated at the age of 14 weeks and again 1 month later with irradiated nontransduced tumor cells (11 mice) or *Ag38*-transduced tumor cells (11 mice); the third group of mice (8 animals) was left untreated and used as control to evaluate spontaneous tumor incidence. In mice vaccinated with *Ag38*-transduced cells, the onset of the first spontaneous mammary tumor was significantly delayed as compared with the control group ($P = 0.033$; Fig. 2*b*), whereas no difference in tumor onset was observed between mice vaccinated with nontransduced N202.1A cells and controls ($P = 0.689$; Fig. 2*a*).

Bacterial DNA has been reported to induce macrophages to secrete

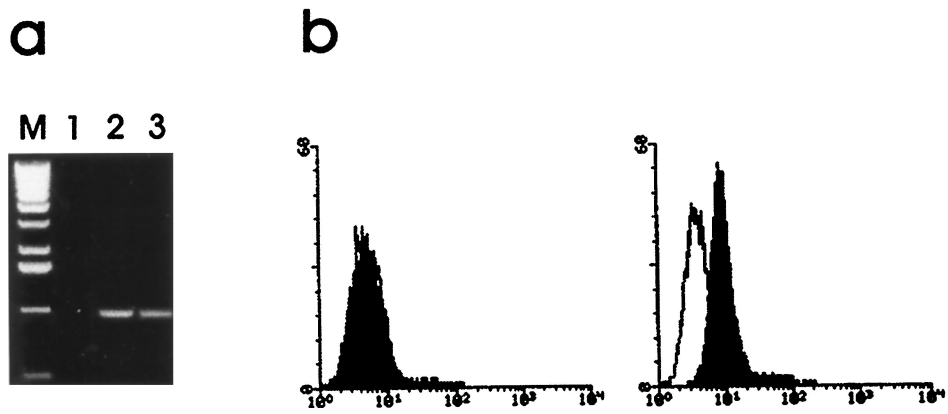

Fig. 1. Expression of *Ag38* in transduced N202.1A cells. *a*, total RNA from mock-transduced cells (*Lane 1*) and from N202.1A cells transduced with pLAg38TMSN vector (*Lane 2*) was assayed by RT-PCR for the presence of *Ag38* mRNA. *Lane 3*, amplification of pLAg38TMSN vector. *M*, molecular weight marker (1-kb ladder). *b*, FACScan analysis of mock cells (*left*) and N202.1A cells transduced with pLAg38TMSN vector (*right*) for cell surface expression of Ag38 protein as detected with mAb HBT12 (relative cell number *versus* log fluorescence intensity). *Open areas*, cells stained with secondary antibody alone.

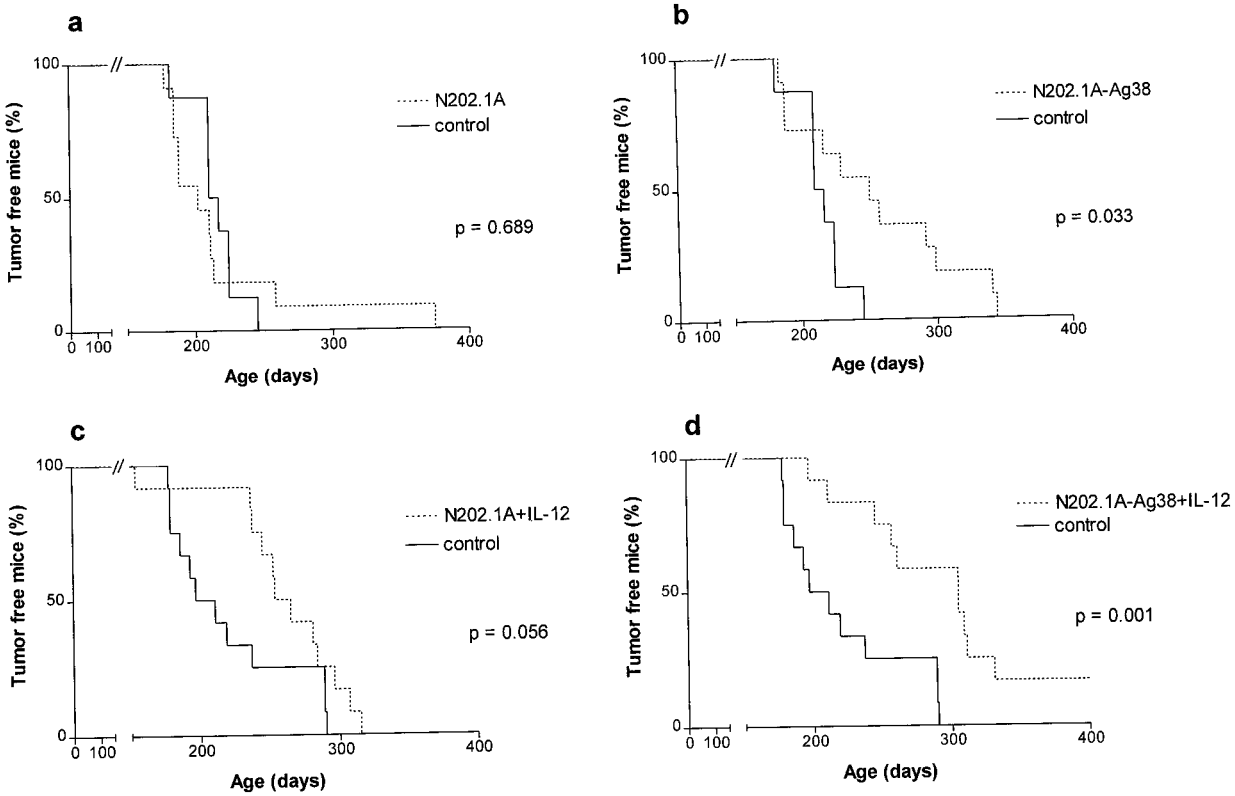

Fig. 2. Spontaneous mammary tumor development in proto-*neu* transgenic female mice vaccinated with nontransduced N202.1A cells. (*a*), *Ag38*-transduced N202.1A cells; (*b*), nontransduced N202.1A cells plus rIL-12 (*c*), and *Ag38*-transduced N202.1A cells plus IL-12 (*d*). *P*s were determined by log-rank test.

IL-12, and indeed, serum of mice maintained under specific pathogen-free conditions revealed detectable levels of IL-12 after injection of bacterial DNA (14). The protection observed with *Ag38*-transduced tumor cell vaccine could be related to a release of IL-12 induced by the presence of *M. tuberculosis* DNA in transduced cells. We investigated whether a systemic coadministration of rIL-12 together with the nontransduced tumor cells could induce the same antitumor response observed using transduced tumor cell vaccine. At the same time, we investigated the antitumor immunity induced by *Ag38*-transduced cancer vaccine plus systemic coadministration of rIL-12. Indeed, systemic administration of rIL-12 was reported recently to potentiate the effects of cancer vaccines engineered to secrete different cytokines (15–17). Thirty-six mice were randomly divided into three groups (12 mice/group), vaccinated with nontransduced or transduced cells as above, and systemically infused with rIL-12 (150 ng over 4 days). The third untreated group was used as control. All mice vaccinated with nontransduced cells plus rIL-12 developed tumors with a slight delay in tumor onset as compared with the control group ($P = 0.056$; Fig. 2*c*), whereas spontaneous tumor development was significantly delayed in mice vaccinated with *Ag38*-transduced cells plus rIL-12, ($P = 0.001$ *versus* control group) with two tumor-free mice at the end of the 400-day observation period (Fig. 2*d*). Moreover, the number of mammary glands with a palpable tumor was reduced in mice vaccinated with irradiated *Ag38*-transduced tumor cells; at 300 days, the mean number of tumors/mouse was 0.91 in this group as compared with 1.5 in mice vaccinated with nontransduced tumor cells plus rIL-12 and 2.08 in the control group.

On the basis of increasing evidence suggesting the importance of

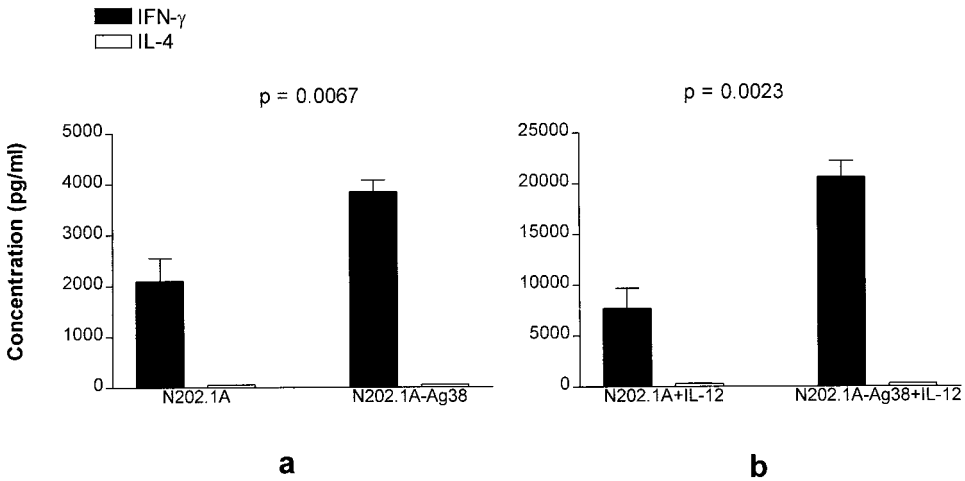

Fig. 3. IFN-γ and IL-4 production by lymphocytes from popliteal lymph nodes of mice vaccinated with nontransduced N202.1A or *Ag38*-transduced N202.1A cells, without (*a*) or with (*b*) rIL-12, and stimulated with immobilized anti-CD3 mAb. Values were obtained by subtracting the cytokine levels in the supernatant of lymphocytes cultured in the absence of anti-CD3 antibody. Experiments were performed two times with comparable results. *P*s were determined by two-tailed unpaired *t* test. *Bars*, SD.

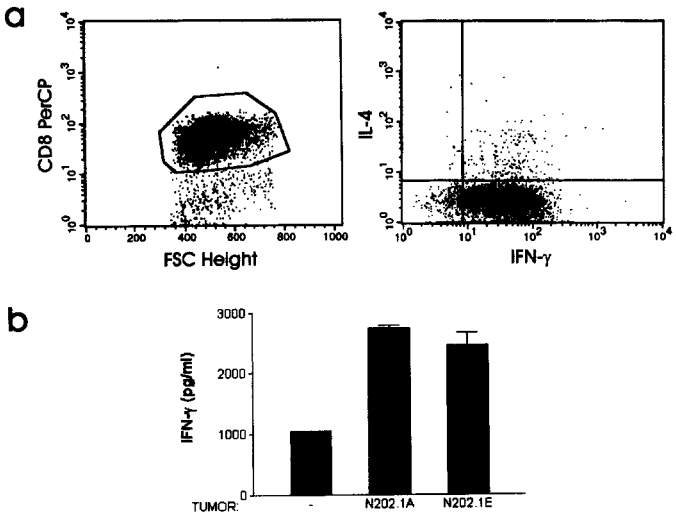

Fig. 4. Characterization of splenocytes obtained from mice vaccinated with *Ag38*-transduced N202.1A cells and restimulated *in vitro* with N202.1A tumor cells and IL-2. *a*, flow cytometric analysis of cells stimulated with immobilized anti-CD3 mAb, in the presence of monensin, and triple-stained with Cy-Chrome-anti-CD8, PE-anti-IL-4, and FITC-anti-IFN-γ mAbs. By gating on CD8-positive cells (94% of total population, *left*), lymphocytes stained with anti-IFN-γ mAb were 89% (*right*). *b*, IFN-γ production measured in supernatant of lymphocytes cultured alone and in the presence of N202.1A or N202.1E tumor cells. *Bars*, SD.

the Th1-type response in the control of tumor growth, we evaluated the Th1- and Th2 responses, represented respectively by IFN-γ and IL-4 production, induced by the different vaccination treatments. Lymphocytes obtained from popliteal lymph nodes of mice footpads injected with *Ag38*-transduced N202.1A tumor cells alone or in the presence of rIL-12 produced significantly higher levels of IFN-γ as compared, respectively, to lymphocytes obtained from mice vaccinated with nontransduced cells alone or in the presence of rIL-12. No production of IL-4 was observed in any group (Fig. 3).

The IFN-γ release induced by vaccination with *Ag38*-transduced cells does not seem to be mediated through the production of IL-12. Indeed, in mice footpads injected with *Ag38*-transduced cells, the administration of an anti-IL-12 antibody did not significantly change IFN-γ production, as compared with the control group (17% inhibition; $P = 0.603$). On the contrary, in mice footpads injected with nontransduced cells and systemically infused with rIL-12, the antibody administration induced, as expected, a significant reduction in IFN-γ production (87% inhibition; $P = 0.0013$).

The induction of a preferential Th1 cytokine secretion pattern by *Ag38*-transduced N202.1A tumor cells is consistent with the absence or a very low titer of antibodies directed against N202.1A-associated antigens in mouse serum samples obtained during the experiments (data not shown). To determine whether the vaccination protocols induced an anti-tumor T-cell response, spleen cells of mice vaccinated with *Ag38*-transduced tumor cells, nontransduced cells plus rIL-12, or nontransduced cells alone were removed 7 days after vaccination and cultured *in vitro* with weekly stimulation with irradiated tumor cells and low-dose IL-2. Only spleen cells of mice vaccinated with transduced tumor cells were able to proliferate in the presence of the tumor cells. After four *in vitro* stimulations, these cells were shown by FACScan analysis to be mainly $CD3^+CD8^+$ and to produce IFN-γ (Fig. 4*a*). Production of IFN-γ by these $CD3^+CD8^+$ cells was not restricted to a response against *HER2/neu*-positive tumors; high levels of IFN-γ were detected in the supernatant of these lymphocytes cultured for 18 h in the presence of *neu*-overexpressing N202.1A tumor, as well as in the presence of the N202.1E tumor cell subline, which does not express the oncoprotein (Fig. 4*b*).

The *in vitro* sensitivity of different transgenic tumor cell lines, *i.e.*, N202.1A, N202.1E, and TT3, to IFN-γ was evaluated in cultures incubated with increasing concentrations of the cytokine. A strong decrease of the proliferation index of the cells was observed (Fig. 5).

DISCUSSION

In the present study, which to our knowledge is the first describing the effect of vaccination in a model of stochastic development of mammary tumors, we show that vaccination of proto-*neu* transgenic mice with *M. tuberculosis*-transduced tumor cells results in a significant delay in tumor onset. DNA of the *M. tuberculosis Ag38* gene used for transduction contains eight CpG motifs. Such CpG DNA motifs have been shown to directly activate monocytes and macrophages to secrete cytokines, especially IL-12 (18), which reaches detectable serum levels in mice, maintained under specific pathogen-free conditions (14, 19). Consistent with these findings, we found detectable levels of serum IL-12 in two of five mice after vaccination with *M. tuberculosis*-transduced cells (data not shown). However, immunization with nontransduced tumor cells plus rIL-12 induced just a slight delay in tumor onset without the recruitment of antitumor T cells, suggesting that the antitumor response involves more than an enhancement of immunity signaled by the CpG DNA motif. Indeed, data obtained by Roman *et al.* (20) using bacterial immunomodulatory DNA sequences showed that these sequences activate the precise cytokine network required to induce an initial burst of IFN-γ in an antigen-independent fashion. In the presence of a protein antigen, the differentiation of naive $CD4^+$ T cells toward the Th1 phenotype can be promoted, leading to a second burst of IFN-γ production, this time in an antigen-dependent fashion. The mechanism of antitumor T-cell recruitment observed with the *M. tuberculosis*-transduced cells remains unclear. The presence of a tuberculosis protein on the surface of irradiated tumor cells can improve their uptake by activated antigen-presenting cells, which might introduce endocytosed tumor antigens into both the MHC class II and class I processing pathways, effecting cross-priming (21, 22). In addition, expression of the mycobacterial gene leading to enhanced Th1 cell maturation might induce the recognition of poorly immunogenic tumor cell antigens and eventually result in a specific antitumor immune response.

The induction of a preferential Th1 response by an endogenously expressed mycobacterial antigen is consistent with findings in other studies (23, 24) and with our previous data obtained in a different mouse strain using murine melanoma cells (9), in which antitumor immunity was induced by the mycobacterial protein, despite the defective expression of costimulatory molecules and of MHC antigens

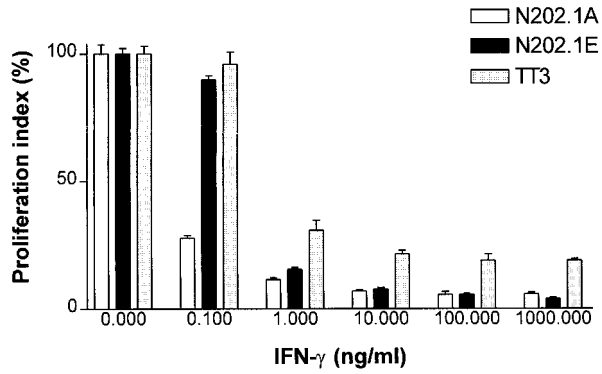

Fig. 5. Effect of IFN-γ on proliferation of N202.1A, N202.1E, and TT3 tumor cells. Proliferation index is expressed as a percentage of absorbance in the absence of IFN-γ. Data are means of quadruplicate determinations; *bars*, SD. Experiments were performed three times with comparable results.

in these cells. The production of IFN-γ by the $CD3^+CD8^+$ lymphocytes and the strong inhibitory effect of this cytokine on the three different transgenic tumor cells suggest that induction of this cytostatic cytokine at the tumor site represents at least one of the mechanism of action of the vaccine. Administration of rIL-12 together with transduced tumor cells resulted in a clear and significant delay in tumor onset with 2 of 12 immunized mice remaining tumor free at the end of the observation period. An adjuvant effect of IL-12, with induction of protective cell-mediated immunity, in a vaccine against Leishmania has been described (25), as well as an increase in both humoral and cell-mediated immune responses in a vaccine against schistosomes (26). In oncology, used alone, IL-12 has been shown to be effective against many murine tumors (27, 28). In proto-*neu* transgenic mice, Boggio *et al.* (29) found that prolonged administration of IL-12 delayed tumor onset and reduced tumor multiplicity in association with deficient peri- and intratumoral angiogenesis and infiltration of reactive cells. IL-12 showed antitumor activity when systemically administrated after tumor challenge in mice vaccinated with *IL-2* gene-transduced tumor cells or in mice vaccinated with B7 costimulatory molecule-expressing tumor cells (15, 30, 31). Recently, an adjuvant effect of IL-12 on vaccination with irradiated SCK tumor cells engineered to secrete granulocyte/macrophage-colony stimulating factor has also been reported (17).

Because a treatment with an anti-IL-12 antibody was not found to significantly modify the IFN-γ production by draining lymph node cells from mice injected with *Ag38*-transduced tumor cells, the effects of *Ag38* transduction on tumor growth seems not mainly mediated by IL-12. Therefore, the cooperativity observed on vaccination between *Ag38* and IL-12 is probably attributable to additional *Ag38*-induced factors that cooperate with IL-12 in inducing greater levels of IFN-γ at the tumor site.

Our data, demonstrating a protective effect of a short-term vaccination protocol that exploits the adjuvant potency of the *M. tuberculosis Ag38* gene and low dose rIL-12, suggest an attractive synergy of these reagents as part of a tumor immunization strategy.

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