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# Evaluation of different plasmid DNA delivery systems for immunization against HER2/*neu* in a transgenic murine model of mammary carcinoma

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**Abstract**

Studies of DNA vaccination against HER2/*neu* showed the effectiveness of immunization protocols in models of transplantable or spontaneous tumors; scarce information, however, has been provided to identify the procedure of DNA administration that more effectively contributes to the activation of immune system against spontaneously arising HER2/*neu*-positive tumors. We compared the effectiveness of different procedures of DNA vaccine delivery (intradermic injection (ID), gene gun (GG) delivery and intramuscular injection (IM) alone or with electroporation) in amurine transgenic model of mammary carcinoma overexpressing HER2/*neu*. We highlighted the role of DNA delivery system in the success of DNA vaccination showing that, among the analysed methods, intramuscular injection of the vaccine, particularly when associated to electroporation, elicits a better protection against HER2/*neu* spontaneous tumor development inducing antibody and cell-mediated immune responsiveness against HER2/*neu* and a Th1 polarization of the immune response.


*Keywords:* DNA vaccination; DNA delivery system; HER2/*neu*; Mammary tumor; Immune response

## 1. Introduction

In the last years experimental evidence has been provided on the potential of genetic vaccination as immunotherapic strategy against cancer. DNA vaccine model is based on the possibility to induce a potent immunity against an antigen expressed by malignant cells. Direct injection into mouse muscle or skin of plasmid DNA encoding a selected antigen results in the expression of the gene product and can elicit an immune response against the antigen of interest [1]. DNA vaccine model represents a promising, practical and effective way to elicit immune responses and, compared to other type of vaccination strategies, has many benefits: DNA vaccines are not MHC-restricted and induce both cellular and humoral immune response, are safe because not infectious, only express the antigens of interest, can be easily manipulated and, finally, are not expensive to produce. Thus, as the goal of a vaccine is to generate a protective immune response specific to the antigen of interest and to sustain this response over a long period of time, DNA vaccination represents a promising, practical and effective way to elicit immune responses against several infectious diseases and against cancer. Many experimental examples show that DNA vaccines directed against tumor associated antigens are able to induce protective immunity to tumor challenge as well as to hamper spontaneous carcinogenesis in murine models [2,3]. Several studies of DNA vaccination have been per-

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formed to find the strategy that more effectively activates the immune system against mammary tumors overexpressing HER2/*neu*, an oncogene coding for a transmembrane protein ($p185^{neu}$) and belonging to the family of tyrosine kinase growth factor receptors. HER2/*neu* gene amplification and consequent over expression of HER2/*neu* receptor have been observed in a significant proportion of human cancers including carcinoma of the breast, prostate, ovary, uterus, stomach and adenocarcinoma of the lung and is intimately associated with malignant phenotype and aggressiveness of the malignancy [4]. We and others [5–20] demonstrated the efficacy of genetic vaccination in different models of transplantable and spontaneously arising tumors overexpressing HER2/*neu* and various approaches of DNA vaccination have been shown to be effective in inducing immunity against this tumor antigen.

An issue in developing tumor DNA vaccines is to design protocols that can be translated from murine models to large animal models and clinical human use without losing their potency [21,22].

Therefore, studies on genetic vaccination against HER2/*neu* are focused on improving DNA vaccines to induce strong effector and memory responses to HER2/*neu.* Attempts to enhance the immune response to DNA vaccines can be performed by using immune and genetic adjuvants or "prime and boost" regimens. The use of cytokines [5,10,15] or other immune-modulating molecules [8,9,11,16] has been indeed proposed to enhance the strength of DNA vaccines against HER2/*neu* and widely studied.

The quality of the immune response elicited by a DNA vaccine is also dependent by the procedure of DNA delivery that influences the mechanisms of DNA uptake, transgene expression, and transgene product processing [23]. The understanding of the immune mechanisms associated to the success of the method of DNA vaccination could provide useful information to determine the requirements of an optimal DNA vaccine. Thus, the role of the DNA delivery system on the outcome of the vaccine should be considered in the elaboration of a HER2/*neu* DNA vaccine. Several DNA vaccines to HER2/*neu* were given by intramuscular injection (IM) [8,12,14,17,18,20] a method that, as recently shown, can be evidently improved by the attracting technology of electroporation of DNA [6]. In some other cases cutaneous delivery, such as intradermal injection [9,10,11,16] or gene gun (GG) delivery [7,24], has been preferred to intramuscular delivery for DNA plasmids administration. Only in few cases, between the cited studies, different systems of vaccination have been compared in the same experimental model [6,9,16] and, with the exception for the comparison of intramuscular vaccine with electroporation [6] these investigations were performed in models of implanted tumors that consist of the challenge of mice with a bolus of tumor cells giving rise to a fast and unnaturally growing tumor. On the contrary, transgenic mice reproduce the more complex spontaneous progression of a preneoplastic lesion and their use provides information that may be more relevant to cancer development in humans where the tumor is initiated by the clonal expansion from a single cell in vivo.

In the present study, with the aim of understanding how DNA delivery system can affect the immune response elicited by a HER2/*neu* vaccine and, consequently, its efficacy in preventing the appearance and the development of HER2/*neu* spontaneous tumors, various type of DNA immunisations were compared in a model of spontaneously arising HER2/*neu*-positive tumors: intramuscular delivery of DNA followed or not by electroporation and cutaneous delivery of the vaccine through intradermal injection or gene gun delivery were used in FVB/*neu*-T transgenic mice and the type of immune response elicited by the vaccines was correlated with their protective potential.

## 2. Materials and methods

### 2.1. Animals

FVB/*neu*-T female mice [25] transgenic for the activated rat HER2/*neu* oncogene (HER2/*neu* oncomice with H-2q FVB/n background) were purchased from Charles River (Hollister, CA, USA) and maintained under specific-pathogen-free conditions in our animal facility. Mice were housed in plastic non-galvanised cages (4–6 mice per cage) maintained at a constant temperature ($20 \pm 1\,^{\circ}C$) and humidity ($50 \pm 5\%$) on a 12 h light/12 h dark cycle and fed with standard pellet food (Nossan, Italy) and tap water "ad libitum".

### 2.2. Plasmids

pCMV-ECDTM plasmid, encoding extracellular and transmembrane region of HER2/*neu* antigen under the control of the CMV early promoter/enhancer, was used for immunizations; pCMV-β-gal plasmid, encoding unrelated antigen (β-galactosidase) and obtained by cloning β-galactosidase sequence in a pCDNA3 vector, was used to treat control mice. Large scale preparation of plasmid DNA was carried out by *Giga kit* (Qiagen, Milan, Italy,) according to the manufacturer's instructions.

### 2.3. Cells

N202/1A is a cloned cell line overexpressing rat HER2/*neu* oncogene that was established in vitro from a lobular carcinoma spontaneously arising in FVB*neu*-N mice [26]. N202/1A cells were cultured in DMEM (Life Technologies, Milan, Italy) penicillin (100 U/ml) and streptomycin (100 μg/ml; penicillin–streptomycin; GIBCO, Milan, Italy) and supplemented with 20% FBS (Life Technologies, Milan, Italy). Before their use for in vitro lymphocyte stimulation assay N202/1A cells were treated wilh mitomycin (60 μg/ml; SIGMA, Milan, Italy) for 30 min.

### 2.4. Immunisation protocol

Two months old FVB*neu*-T female were randomly selected for immunisation with DNA vaccine (pCMV-ECDTM, pCMV-β-gal) by intramuscular injection without or with electroporation (IM or IM + E), intradermic injection (ID) or gene gun; animals were routinely immunised by three administrations carried out at 8, 12 and 16 weeks of age. For IM injection each mouse was anaesthetised and DNA was delivered into quadriceps muscle previously exposed; each mouse received 100 μg DNA in 100 μl saline solution. For IM + E injection 50 μg of DNA dissolved in $H_2O$ containing 6 mg/ml L-glutammate and 150 mM NaCl were given to each animal, previously anaesthetised, through two injections in the tibial muscle followed by three electric pulses (field strength = 200 V/cm; pulse length = 25 ms; ECM 830 field generator, BTX Division, Genetronix, San Diego, CA, USA). For ID injection animals were anaesthetised and received 100 μg DNA in 100 μl saline solution into the derma of the back. For gene gun injection DNA-coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (Bio-Rad Laboratories Inc., Hercules, CA, USA) with a discharge pressure of 400 psi; at every administration each mouse received 2 μg DNA. Protocols and regimens of vaccination used were previously established so that the amount of DNA injected differs for every method of DNA delivery depending on the different efficacy of in vivo transfection and on the type of procedure typical of every technique used.

### 2.5. Analysis of tumor growth

After immunisation, treated and control mice were controlled twice per week to evaluate incidence and growth of tumors. Mice with no evidence of tumor at the end of the observation period were classified as tumor-free whereas mice with a tumor of at least 3 mm mean diameter were classified as tumor bearers. Number of tumor masses/animal was also registered. All mice bearing neoplastic masses exceeding 10 mm mean diameter were killed for humane reasons.

### 2.6. Preparation and culture conditions of spleen cells

Spleen was teased through a 60 mesh sieve in $Ca^{2+}$and $Mg^{2+}$ free phosphate buffered saline (PBS, GIBCO, Gaithersburg, MD, USA) solution. Spleen cells were then repeatedly washed with PBS and resuspended in RPMI 1640 (Life Technologies, Milan, Italy) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), 10% FBS and 50 U/ml interleukin-2 (IL-2; Chiron Corporation, Emeryville, CA, USA).

### 2.7. Cytotoxic assay

Splenocytes obtained from mice spleen were incubated at 37 °C and 5% $CO_2$ in RPMI medium containing 10% FCS in the presence of mitomycin-treated N202/1A tumor cells as stimulators (20:1 ratio stimulators:lymphocytes) for 5 days. Cytotoxic assay was performed using a fluorimetric method as previously reported [27]. Briefly, a stock solution of carboxyfluorescein diacetate (c'FDA, Molecular Probes, OR, USA) (20 mg/ml acetone, stored at −20 °C) was diluted in PBS to give a final concentration of 75 μg/ml. N202/1A tumor cells were washed twice with PBS and then labelled with c'FDA by resuspending the cells in 1 ml working solution and incubating at 37 °C in a humidified 5% $CO_2$ incubator for 30 min. Target cells were then washed three times in PBS containing 1% BSA (SIGMA) and suspended in RPMI + 10% FCS at a concentration of $0.5 \times 10^5$ $ml^{-1}$. $0.5 \times 10^4$ c'FDA-labelled tumor target cells were incubated with effector spleen cells in 200 μl total volume in 96-well round microtiter plates (Nunc, Roskilde, Denmark). Effector: target cell ratios from 100:1 to 12.5:1 were tested in triplicate. The plates were kept at 37 °C in a humidified, 5% $CO_2$ incubator for 3 h and then centrifuged at 700 g for 5 min. The supernatant was separated from the cellular fraction by rapidly inverting the plate and flicking the supernatants out. Then, 100 μl of 1% triton X100 in 0.05 M borate buffer, pH 9.2 was added to each well. The plate was kept for 20 h at 4 °C to allow for solubilization and then was read for fluorescence with a 1420 VICTOR$^2$ multilabel counter (Wallac, Turku, Finland). The percentage of specific lysis was calculated as follows:

$$\%\text{Specific lysis} = \left[\frac{F_{\text{med}} - F_{\text{exp}}}{F_{\text{med}}}\right] \times 100$$

where $F$ represents the fluorescence of the solubilized cells after the supernatant has been removed; med = $F$ from target incubated in medium alone; and exp = $F$ from target incubated with effector cells.

### 2.8. Intracellular cytokine staining

Splenocytes obtained from mice spleen were incubated overnight at 37 °C and 5% $CO_2$ in RPMI medium containing 10% FCS in the presence of mitomycin-treated N202/1A tumor cells as stimulators (20:1 ratio stimulators lymphocytes) for 2 days; cells were harvested and stained in PBS buffer containing 5% FCS and 0.01% $NaN_3$, with anti-CD4 or anti-CD8 monoclonal antibodies (BD Pharmingen) FITC-conjugated; cells were then fixed in 2% formaline, successively stained in a PBS buffer containing 5% FCS and 0.05% Saponin (SIGMA) with anti-IL10, anti-IL4 or anti-IFN-γ (CALTAG) PE-conjugated, and finally analysed by a Coulter XL flow cytometer.

### 2.9. Rat-pl85$^{neu}$ specific antibody analysis

Sera of treated and control mice were harvested 2 weeks after the end of immunisation protocols and stored at −80 °C until use. In order to assess the presence p185$^{neu}$ specific antibody the ability of sera to bind p185$^{neu}$ was evalu-

ated by flow cytometry. $2 \times 10^5$ N202/1A cells were washed twice with cold PBS supplemented with 2% BSA and 0.05% sodium azide. Cells were then stained in a standard indirect immunefluorescence procedure with 50 μl of 1:10 dilution in PBS-azide-BSA of control or immune sera. A fluorescein-conjugated rabbit anti-mouse Ig (Calbiochem, Milan, Italy) or anti-mouse IgGl, anti-mouse IgG3, anti-mouse IgG2a and anti-mouse IgG2b (Caltag Laboratories, Burlingame, CA, USA) were used as second step Ab. The cells were resuspended in Isoton II (Coulter, Hialeah, FL, USA) and evaluated through a Coulter XL flow cytometer. The specific 202/1A binding potential (SBP) of the sera was calculated as follows: [(% positive cells with test serum) (fluorescence mean) × serum dilution], as previously described in detail [28].

### 2.10. Adoptive transfer of sera

In order to test the capacity of anti-p185$^{neu}$ sera to interfere with the development of p185-overexpressing tumor cells, 2 months old FVB*neu*-T female received sera from treated and control mice. One hundred fifty micro litre of pooled sera of mice belonging to each one of the treatment groups were injected intraperitoneally in each animal (five animals/treatment sera). Twenty-four hours after the treatment with sera mice were s.c. challenged with $10^5$ N202/1A tumor cells and successively monitored to register the kinetic of development of injected tumors.

### 2.11. Statistical analysis

Differences in tumor incidence were evaluated by the Mantel–Haenszel log-rank test; differences in tumor multiplicity were evaluated by Student's *t*-test. Differences in immune parameters were evaluated by ANOVA followed by Student–Newman–Keuls post-hoc test when appropriate. Differences were considered statistically significant when $p < .05$.

## 3. Results

### 3.1. Effect of the vaccine on the kinetics of growth of spontaneous mammary tumors in FVBneu-T mice

Immunisation experiments were performed in order to analyse the effect of the vaccine on the kinetics of growth of spontaneous mammary tumors in FVB/*neu*-T oncomice following the different protocols of vaccine administration. FVB*neu-T* mice were preventively immunised starting at 8 weeks of age, when they are still free of palpable tumors and hyperplasia of the mammary tissue is not yet present ([29] and data not shown), through gene gun, intradermal injection or intramuscular injection alone or followed by electroporation (IM + E) with a plasmid encoding the extracellular and transmembrane domain of HER2/*neu* receptor (pCMV-ECDTM).

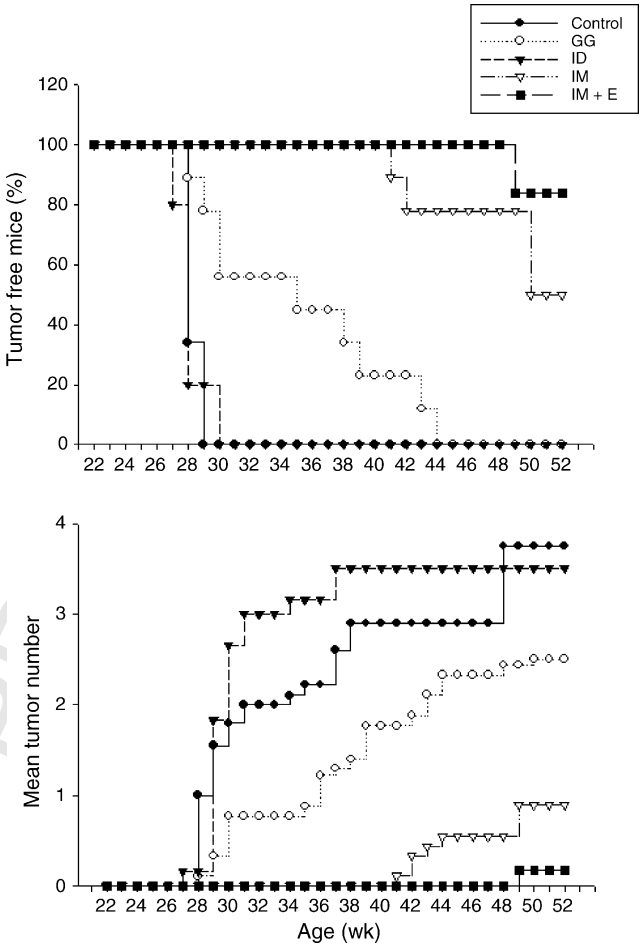



Fig. 1. Inhibition of mammary carcinogenesis in HER2/*neu* transgenic mice vaccinated with pCMV-ECD-TM plasmid using different systems of DNA delivery (ID, GG, IM, IM + E). Percentages of tumor-free mice were calculated as cumulative number of tumor bearer and tumor free mice (top); mean number of palpable mammary carcinomas per mouse was calculated as cumulative number of incident tumors/total number of mice (bottom). All groups included 10 animals/experiment. Data shown are of one from two experiments performed with the same number of animals. Difference in tumor incidence, as assessed by the Mantel–Haenszel log-rank test, was significant between GG ECDTM vs. control ($p < 0.001$), IM ECDTM vs. control ($p < 0.0001$), IM + E ECDTM vs. control ($p < 0.0001$), and GGECDTM vs. IM ECDTM or IM + E ECDTM ($p < 0.0001$). At Week 52 of age, tumor incidence was significantly different between IMECDTM + E vs. IM ECDTM ($p < 0.01$). Differences in tumor multiplicity, as assessed by Student's *t*-test, were significant between IM ECDTM or IM + E ECDTM vs. control from Week 29 ($p < 0.02$).

Graph of Fig. 1 shows that the treatment with pCMV-ECDTM through different plasmid injection procedures differently affected the incidence and the growth of spontaneous tumors in FVB*neu*-T transgenic mice. In controls, the first tumor mass appeared at around Week 28 of age and, by Week 30, 100% of mice were tumor bearers. The ID administration of the vaccine did not substantially modify either the course of tumor appearance or their development when compared to control animals. Similarly, in GG treated animals the vaccine did not delay the time of appearance of the first tumor mass, although the number of tumor bearing mice increased with a

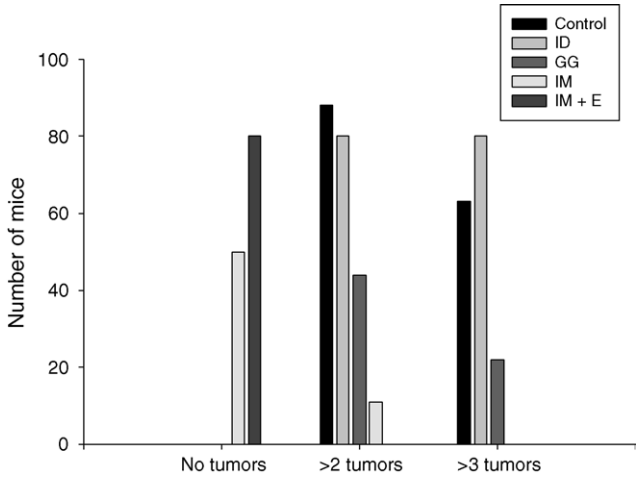



Fig. 2. Number of mammary carcinomas in FVB*neu*-T transgenic mice vaccinated with pCMV-ECDTM plasmid following different system of DNA delivery. The percentage of tumor-free mice and of mice having 2> tumor or 3> tumors is shown.

slower kinetic in comparison with control mice, and only at Week 44 of age 100% of mice presented tumors ($p < 0.001$ versus control group). In animals which received DNA by IM injection, the time of tumor onset was drastically delayed: in IM group first tumors appeared in only 20% of mice at around Weeks 40–42, and 45% of mice were still tumor free at Week 52 ($p < 0.0001$ versus control group). Electroporation significantly improved the outcome of IM delivered vaccine so that at Week 48 of age 100% of mice were still tumor free and at Week 52 of age only the 20% of mice presented tumors ($p < 0.0001$ versus control group; $p < 0.01$ versus IM). The kinetics of tumor appearance in IM or IM + E treated mice was significantly different from that of GG treated animals ($p < 0.0001$). The protective effect of the vaccine was assessed also by monitoring the variation of mean number of tumor masses per mouse. As shown in Fig. 1 (bottom) and in Fig. 2, the reduction of tumor incidence obtained in IM immunized mice was associated to a decrease of the number of tumor masses arising in treated mice. In both IM and IM + E mice groups, a significantly lower tumor multiplicity was found in comparison with control animals ($p < 0.02$). The mean number of tumor masses in IM + E mice group was significantly lower when compared to IM mice group from Week 44 of age ($p < 0.05$). In all ID treated mice a high number of masses appeared so that 80% of animals presented more than three tumors, as control mice. On the contrary, in IM and IM + E groups the few tumor bearing mice did not develop more than one tumor mass. GG injection leaded to an intermediate result since only 20% of mice developed more than three masses and the mean number of tumors/mouse remained lower than control and ID injected animals.

### 3.2. Production of antibodies against rat-p185$^{neu}$

Humoral immunity elicited by the vaccine was analysed in the different groups of treatment. Sera of treated and

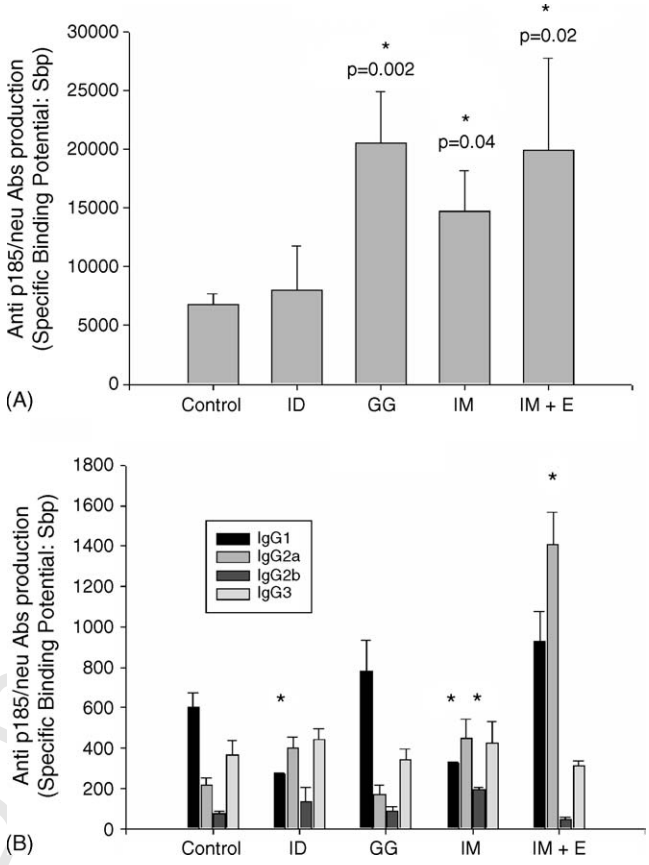



Fig. 3. Humoral immunity in FVB*neu*-T transgenic mice vaccinated with pCMV-ECDTM plasmid following different systems of DNA delivery. Specific binding potential of sera from treated and control animals to p185 protein (A) and anti-p185$^{neu}$ IgGs isotypes (B) are shown. Sbp was evaluated by flow cytometry after indirect immunofluorescence. Asterisk indicates statistical significant difference vs. control.

control mice were harvested 2 weeks after the end of the immunization protocol and successively analysed to assess the induction of a specific humoral immune response. As shown in Fig. 3(A), tumor-specific antibodies were observed in all not vaccinated FVB/*neu*-T oncomice that naturally develop a humoral immunity against rat HER2/*neu* transgene. In particular our analysis showed that immunoglobulines of IgGidiotype released in control mice were characterised by a prevalence of IgGl subclass, that is typical of a Th2-type immunity. Except for ID injection, all other treatments significantly increased the titre of anti-HER2/*neu* antibodies although the amount of produced antibodies only partially correlated with the outcome of vaccination. On the contrary the quality of the humoral response seems to be important for the efficacy of the vaccine. Fig. 3(B) shows the IgG isotype distribution in mice immunised with different delivery systems.

Interestingly, GG vaccination did not change the IgG pattern observed in not immunised mice; in IM sera, differently, IgG1 titre decreased in comparison to control mice ($p < 0.05$) while a prevalence of Th1-like IgGs (IgG2a, IgG2b, IgG3) was observed. IM + E vaccination promoted a high increase

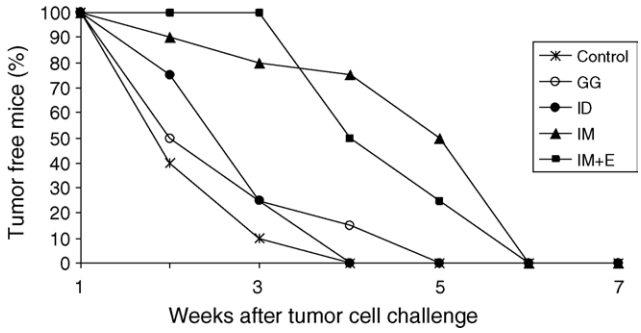



Fig. 4. In vivo anti-tumor effect of anti-p185 sera from immunized mice. The capacity of anti-p185$^{neu}$ sera to interfere with the development of p185-overexpressing 202/1A tumors cells is shown. Sera from vaccinated and control mice were i.p. injected in 2 month old FVB*neu*NT mice; 24 h after mice were s.c. challenged with $10^5$ N202/1A tumor cells. Difference in tumor incidence, as assessed by the Mantel–Haenszel log-rank test, was significant between GGECDTM vs. control ($p < 0.01$), IM ECDTM vs. control ($p < 0.01$), and IM + E ECDTM vs. control ($p < 0.01$).

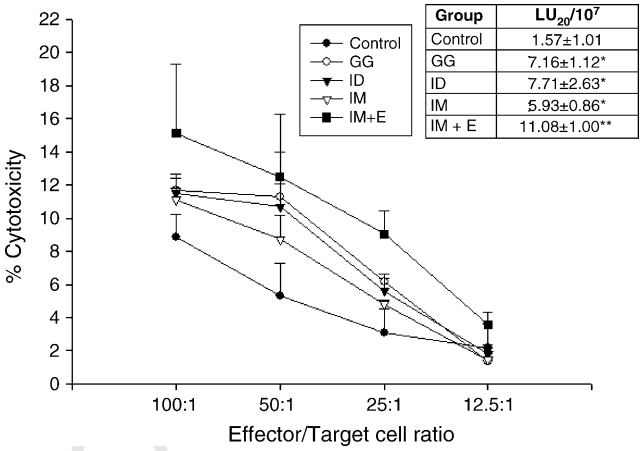
of IgG2a ($p < 0.01$ versus control and IM), suggesting a correlation between the effectiveness of the vaccine and the induction of a Th1-like immunity.

### 3.3. Adoptive transfer of immune sera

The ability of sera obtained from HER2/*neu* immunised mice to protect against tumor growth in vivo was assessed by administrating sera from treated mice to untreated animals that received a s.c. injection of N202/1A tumor cells. As shown in Fig. 4, tumor-specific sera were able to delay the time of appearance and development of N202/1A tumors. Sera from GG treated mice lightly protected animals from the growth of N202/1A tumors ($p < 0.01$ versus control group). Sera harvested from intramuscularly immunised mice, both without or with electroporation, more evidently protected animals from N202/1A challenge ($p < 0.01$ versus control group). The protection afforded by sera from IM and IM + E mice did not show statistically significant difference.

### 3.4. Cytotoxic activity and cytokine production

In order to analyse the cellular immunity induced by the different methods of immunization, spleen cells from immunized mice were analyzed for their cytotoxicity and cytokine production after in vitro incubation with HER2/*neu* overexpressing tumor cells. As shown in Fig. 5 all DNA vaccination methods against HER2/*neu* increased anti-HER2/*neu* specific cytotoxic activity of lymphocytes in comparison with control group ($p < 0.05$). In particular, the efficacy of the vaccine was evident in the case of IM + E vaccinated mice where the cytotoxicitv was 10-fold increased compared to controls ($p < 0.05$); a less evident, but still significant augmentation of cytotoxic activity in comparison with the other groups of mice was observed ($p < 0.05$). In all groups the activation of an HER2/*neu*-specific immunity was associated to a slight




| Group | $LU_{20}/10^7$ |
|---|---|
| Control | 1.57±1.01 |
| GG | 7.16±1.12* |
| ID | 7.71±2.63* |
| IM | 5.93±0.86* |
| IM + E | 11.08±1.00** |

Fig. 5. Cell-mediated cytotoxic activity in HER-2/*neu* transgenic mice vaccinated with pCMV-ECDTM plasmid following different systems of DNA delivery. Cytotoxicitv was performed through a fluorimetric assay. Data are reported as percent of lysis obtained at different E:T ratios and as number of lytic units ($LU_{20}/10^7$). Difference in cytotoxicity was significant between GG, ID, IM or IM + E vs. control group (*) $p < 0.05$ and between IM + E vs. GG, ID, or IM groups (**) $p < 0.05$.

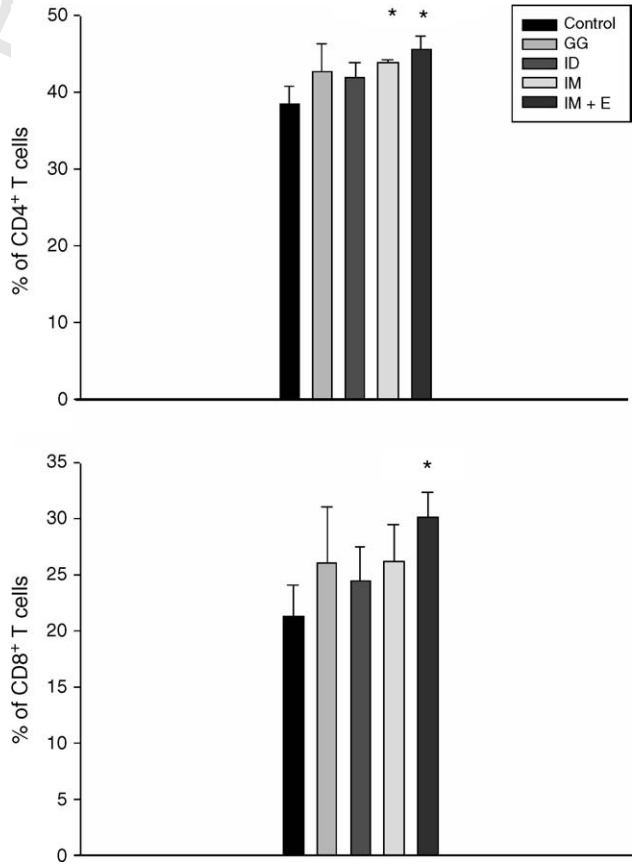



Fig. 6. Effect of different systems of DNA delivery on the percentage of CD4 or CD8 T cells. Spleen cells from mice vaccinated using different systems of DNA delivery were stained with anti-CD4 or anti-CD8 monoclonal antibodies and analyzed at a flow cytometer. Difference in the percentage of CD4 T cells was significant between IM or IM + E and control group ($p < 0.01$). Difference in the percentage of CD8 T cells was significant between IM + E and control group ($p < 0.03$).

increase of the percentage of CD4+ and CD8+ T cells in comparison with control group, that, however, was significant only for IM and IM + E groups in the case of CD4 cells ($p < 0.0l$) and only for IM + E group for CD8 cells ($p < 0.03$) (Fig. 6). As shown in Fig. 7, a significant augmentation of IFN-γ production by CD8 T cells was observed for IM and IM + E groups with respect to controls ($p < 0.05$). In vaccinated mice, compared to controls, an increased number of IL10-producing CD4 T cells was found in IM and IM + E groups; no delivery system stimulated the production of the Th2-like cytokine IL4 by CD4 T cells.

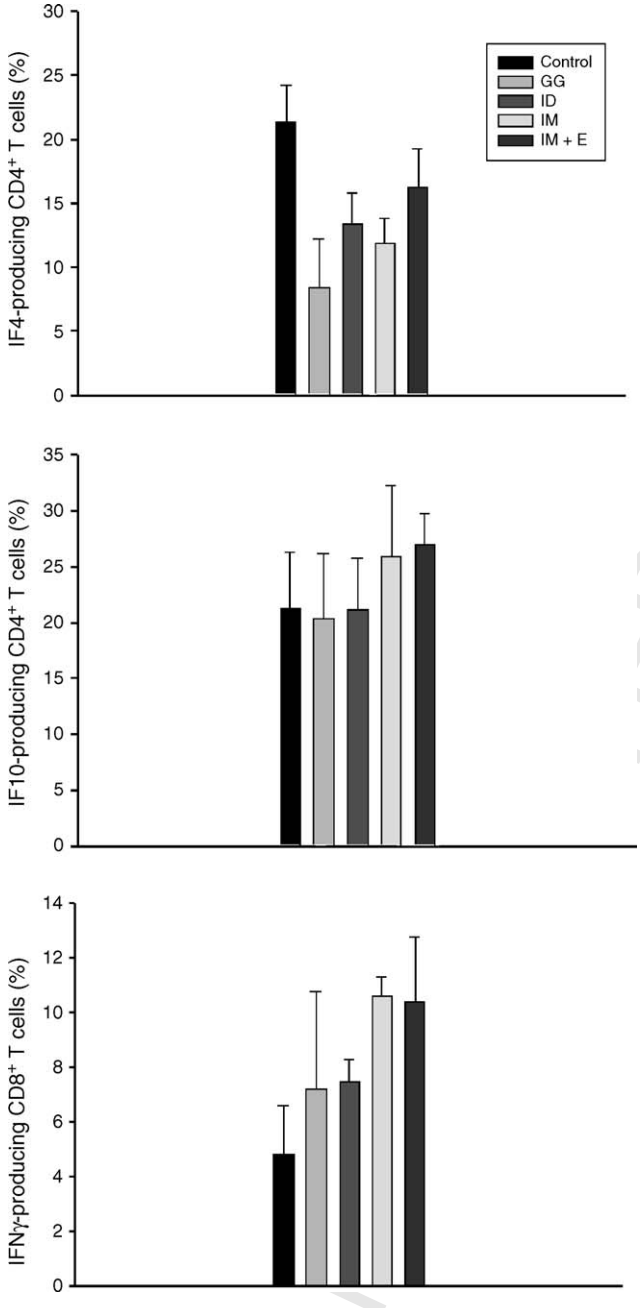



Fig. 7. Intracellular cytokine staining in HER2/*neu* transgenic mice vaccinated with pCMV-ECDTM plasmid following different systems of DNA delivery. The percentage of CD8 T cells containing IFN-γ. and the percentage of CD4 T cells containing IL10 or IL4 was determined through double-staining flow cytometry after in vitro lymphocyte incubation with mitomycin-treated N202/lA tumor cells. All data are expressed as percentage of positive cells. Difference in the percentage of IFN-γ-producing CD8 T cells was significant between IM or IM + E and control group ($p < 0.05$).

## 4. Discussion

By virtue of its versatility and efficacy in inducing immunity against tumor antigens DNA vaccination represents a suitable approach for the treatment of tumors [1–3]. Many strategies of DNA vaccination against HER2/*neu* have been proposed by our and other groups to prevent the development of HER2/*neu*-positive mammary carcinomas in various experimental models including transplantable and spontaneously arising transgenic tumors [5–20]. In view of the translation of DNA vaccination from murine to large animal models and clinical human trials the goal of all investigators is to identify the way to improve the potency of HER2/*neu* DNA vaccines. The use of different adjuvants in association with DNA vaccines proposed against HER2/*neu* tumor antigen has been studied [5–20], but prior to modify vaccine formula, the vaccine could be improved by optimising the procedure of DNA delivery to favour the process of DNA uptake by host cells [23].

Several studies demonstrated the efficacy of various systems of DNA immunisation in animal models and highlighted that diverse protocols of DNA immunisation result in the activation of different immune responses and exert different protective effects [30–37]. An interesting study on the outcome of various protocols of DNA vaccine delivery showed, for example, that DNA immunisation via intramuscular and intradermal routes elicits immune responses of different magnitude and duration. Ito et al. [32] demonstrated that while intradermal injection of DNA induces an higher Ab and CTL response, intramuscular delivery of the vaccine provides a persistent antigen production of the antigen by long-term transfected host cells that supports a longer lasting, although weaker, immunity.

In the case of HER2/*neu* DNA vaccination no comparison of the different systems of DNA vaccine delivery via intramuscular or cutaneous administrations proposed for the prevention of spontaneous carcinogenesis in transgenic models of mammary carcinoma has been done.

The present study, where intramuscular delivery of DNA, followed or not by electroporation, was compared with its cutaneous delivery through gene gun or intradermal injection, has been performed in the transgenic model of FVB/*neu*-T mice that develop spontaneous mammary carcinomas following the overexpression of HER2/*neu* oncogene in mammary tissue of sexually mature females [25]. In these mice, where an immune response specific for HER2/*neu* expressing tumors, although not protective, is naturally elicited by the overexpression of the oncogene [29], DNA vaccination through intramuscular delivery resulted promis-

ing [17,19] to hamper spontaneous carcinogenesis. Thus, we performed this study in order to understand in which terms the method of DNA delivery is relevant to induce a protective immunity in FVB/*neu*-T mice and if the success of intramuscular immunisation was correlated with the induction of particular immune mechanisms against HER2/*neu*.

Data presented here confirmed that in this transgenic model of spontaneous mammary carcinoma the efficacy of pCMV-ECDTM DNA vaccine against HER2/*neu* is influenced by the method of release of DNA. We showed that the vaccine delivery methods analysed elicited diverse immune mechanisms that differently prevented the appearance and the development of spontaneous mammary carcinomas. In particular, IM + E injection that, in another model of spontaneous carcinogenesis, was recently shown to be successful in inhibiting multifocal preneoplastic lesions already present at the time of vaccination, in our model of tumor prevention resulted in the best antitumoral effect and in the generation of a Th1-type immune response.

In FVB*neu*-T HER2/*neu* mice [25] the process of carcinogenesis induced by the overexpression of activated rat-p185 in all mammary glands leads, by 30 weeks of age, to the appearance of clinical tumors with a fast kinetic of growth in all mice. When intradermically administered, pCMV-ECDTM vaccine did not altered substantially the kinetic of tumor growth and when given through gene gun delivery exerted only a partial protective effect. Differently, intramuscular immunization resulted in the best outcome since in IMmice pCMV-ECDTM vaccine was able to drastically delay both tumor onset and the mean number of tumor masses arising in treated mice. The efficacy of IM delivered vaccine was significantly improved by the procedure of electroporation that further delayed the time of appearance of tumors and increased from 45 to 80%, the percentage of mice that were still tumor free at 1 year of age.

In order to correlate the effects of the different delivery systems to the immune response, we performed some experiments to investigate humoral and cellular immunity elicited by the vaccine in the different groups of treatments.

Anti-HER2/*neu* antibodies were found in the sera of all control and treated mice. In particular, all treatment, except for ID vaccination, induced a significant increase of the titre of anti-HER2/*neu* antibodies. Moreover, while the amount of produced antibodies only partially correlated with the outcome of vaccination, the quality of humoral response seems to be determinant for the success of vaccination. The efficacy of IM or IM + E vaccine treatments were associated to the production of a Th1 pattern of IgGs. The role of tumor-specific antibodies in the protection of animals against tumor development was also demonstrated by the fact that the adoptive transfer of immune sera hampered the growth of HER2/*neu*-positive tumors injected s.c. in not immunized mice. As it was argued elsewhere [38], is plausible that HER2/*neu*-specific antibodies acquire a relevant protective role, since HER2/*neu* oncoprotein, besides being a target tumor antigen, is a receptor involved in regulation of cell proliferation.

A tumor specific cellular immunity was also elicited by all treatments in vaccinated mice. The augmentation of IFN-γ production by CD8 T cells, that resulted statistically significant for IM and IM + E groups, was associated to an increase of the in vitro cytototoxic activity of lymphocytes from all vaccinated mice. Although in all groups the capacity to kill HER2/*neu* expressing tumor cells was significantly increased compared to control mice, the influence exerted by electroporation on intramuscular injection of the vaccine was, as expected, clearly evident with increases of cytotoxicity significantly higher than those obtained by IM immunization alone. Surprisingly in lymphocytes obtained from mice of IM and IM + E groups, where the vaccine exerted the best protective effect, we observed an increased production of IL10 by CD4 T cells. IL10 is a cytokine with pleiotropic effects that is mainly known for its potent immunosuppressive properties, but, in some instances, it has been shown to paradoxically augment tumor immunity [39]. In particular, IL10 producing CD4 T cells have been demonstrated to exert antitumor effects acting as regulatory cells rather than typical Th2 cells [40]. Interestingly, in a recent study [41] we observed a significant production of IL10 by CD4 T cells associated to the adjuvant action of an immunostimulant molecule used to improve the efficacy of a HER2/*neu* DNA vaccine in the same model of transgenic mice. These observations, through needing to be confirmed by further studies, suggest that the activation of IL10-producing CD4 T cells could be a determinant event to build an immune response able to hamper spontaneous carcinogenesis in FVB/*neu*-T trangenie mice. In conclusion, intramuscular delivery was confirmed the better route to elicit a complete immune response of Th1 type and mediated by different mechanisms that conferred a high protective potential to the vaccine. The efficiency of intramuscularly injected vaccine was clearly improved by electroporation procedure that, favouring the uptake of DNA into host cells and the expression of the encoded protein [42], increased the intensity of the immune response. On the contrary, cutaneous administrations of the vaccine were not so successful: the Th1-like immunity induced by ID injected vaccine was missing a strong humoral response and was quantitatively too low to oppose tumor development; GG delivery, although is an efficient procedure that require a minimal amount of DNA to induce an immune reaction against the tumor, mainly elicited a Th2-like immune response that only partially protected mice from carcinogenesis.

The identification of the best procedure of DNA delivery represents a basic step for the optimisation of genetic immunization protocols that can be further improved by the use of immune adjuvant approaches to develop vaccines that could be used also in immune-depressed individuals such as aged subjects where the ability of immune system to hamper the development of a tumor is impaired [24,43].

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