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Local delivery of recombinant vaccinia virus encoding for neu counteracts growth of mammary tumors more efficiently than systemic delivery in neu transgenic mice

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Abstract

Recombinant vaccinia virus has been widely employed as a cancer vaccine in several clinical trials. In this study we explored, employing BALB/c mice transgenic for the rat neu oncogene, the ability of the recombinant vaccinia virus neu (rV-*neuT*) vaccine to inhibit growth of neu+ mammary carcinomas and whether the efficacy of vaccination was dependent on: a) carcinogenesis stage at which the vaccination was initiated; b) number of vaccinations and c) route of delivery (systemic vs local). BALB-*neuT* mice were vaccinated one, two and three times by subcutaneous (s.c) and intramammary gland (im.g) injection with rV-*neuT* or V-wt (wild type vaccinia virus) starting at the stage in which mouse mammary gland displays atypical hyperplasia, carcinoma *in situ* or invasive carcinoma. We demonstrated that vaccination using rV-*neuT* was more effective when started at an earlier stage of mammary carcinogenesis and after three vaccinations. im.g vaccination was more effective than s.c vaccination in inhibiting mammary carcinogenesis, eliciting anti-Neu antibodies, increasing anti-Neu IgG2a/G3 isotypes and inducing antibodies able to trigger mammary tumor cell apoptosis and antibody dependent cellular cytotoxicity. The better protective ability of rV-*neuT* im.g vaccination was associated with its capacity to induce a superior degree of *in vivo* mammary cancer cell apoptosis. Our research suggests that intratumoral vaccination using recombinant vaccinia virus could be employed to increase the activity of a genetic cancer vaccine. This study may have important implications for the design of cancer vaccine protocols for the treatment of breast cancer and of accessible tumors using recombinant vaccinia virus.

Keywords

cancer vaccine; intratumoral vaccination; recombinant vaccinia virus; serum antibodies

Introduction

Attenuated vaccinia virus delivery produces a mild infection in humans, which protects against smallpox caused by the variola virus, due to its ability to provide long-term immunity against the natural form of the virus [1, 2]. The ability of vaccinia virus to insert and express foreign genes encoding for weak immunogenic proteins has led to its use as a delivery vehicle for cancer and infectious diseases vaccines in experimental models [3–5]. Engineered attenuated recombinant vaccinia virus has now been widely employed as a cancer vaccine in a large number of clinical trials. The results of these trials demonstrated that vaccinia virus infection upon vaccination was safe and that a specific humoral or T cell response against the foreign inserted tumor associated antigen could be induced in several cancer patients [3, 5–14]. Vaccination with recombinant vaccinia virus can be achieved by systemic or local intratumoral injection [3, 6–19]. Systemic vaccination employs subcutaneous (s.c), intradermal, or intramuscular delivery [20, 21]. Although the majority of anti-cancer vaccine strategies employ systemic vaccination, recent data support the effectiveness of the intratumoral vaccination both in human and experimental models [11, 13, 15–25]. Recently, it was demonstrated that the antitumor activity induced by intratumoral vaccination with an avipox virus expressing carcinoembryonic antigen (CEA) and multiple costimulatory molecules was superior to that induced by systemic (subcutaneous) vaccination in CEA-transgenic mice [21]. A prerequisite to employing intratumoral vaccine therapy is the simple access for antigen delivery to the tumor site. Among others, mammary cancer is a typical accessible primary tumor. BALB-*neuT* mice that are transgenic for the neu oncogene are a suitable animal model to study the efficiency of Her-2/neu cancer vaccine in counteracting autochthonous mammary carcinogenesis [26, 27]. Tumor vaccine studies have used the BALB-*neuT* model in the past, which included different delivery strategies employing DNA [28], synthetic peptides [29], adenoviral vector [30, 31] or a cell-based vaccine [32, 33]. These vaccines have been shown to protect BALB-*neuT* mice to a variable degree from neu oncogene-induced tumorigenesis. The stepwise progression of mammary carcinogenesis in BALB-*neuT* allows one to begin the vaccination based on the progressive stage of the disease. BALB-*neuT* mice exhibit reproducible transition from normal epithelium to atypical hyperplasia (week 6) and to multifocal breast carcinoma that becomes palpable around week 16 [28].

To our knowledge there are no studies that employed recombinant vaccinia virus as a vaccination vehicle to deliver high recombinant protein levels of Her-2/neu in BALB-*neuT* mice. In this report we explored the mammary tumor inhibitory ability of the recombinant vaccinia virus neu (rV-*neuT*) vaccine in BALB-*neuT* mice. In addition, we investigated whether the efficacy of vaccination was dependent on the carcinogenesis stage at which the vaccination was initiated as well as the usefulness of multiple rV-*neuT* injections. This study compares the antitumor effect induced by systemic vs local route of rV-*neuT* administration, by employing subcutaneous (s.c) vs intramammary gland (im.g), respectively. This study may have important implications for the design of cancer vaccine protocols for the treatment of breast cancer and of accessible tumors using recombinant vaccinia virus.

Materials and Methods

Antibodies, peptides and cells

Synthetic peptides located in the extracellular (Neu 15.3, aa 66–74; Neu 42, aa 169–183; Neu 98, aa 393–407; Neu 141, aa 566–580; Neu 156, aa 626–640), transmembrane (Neu 166, aa 666–680) domains of rat Neu sequence [34, NCI, PubMed Accession 1202344A] were previously described [33]. Neu-overexpressing BALB-*neuT* mammary cancer cells (H-2^d) (TUBO) were previously described [27]. NIH3T3 cells encoding normal rat Neu (LTR-Neu) have been previously characterized and kindly provided by Dr. Eddi Di Marco (Istituto Tumori di Genova) [35]. Polyclonal rabbit anti-neu antibody Ab1 (PC04) was purchased from Oncogene Science (Cambridge, MA). Rabbit polyclonal antibody recognizing the activated cleaved caspase-3 was purchased by Cell Signaling Technology (Beverly, MA; catalog #9661).

Poxviruses

The recombinant vaccinia virus encoding the *neu* oncogene was designated rV-*neuT* (vT67RR-1-1, original lot from Therion Biologics Corp: #SC012197). It encodes the full length activated rat *neu* oncogene [34, NCI, PubMed Accession 1202344A]. The wild type control vaccinia virus was designated V-wt (original lot from Therion Biologics Corp: #062797-NYCBH). Therion Biologics Corp. (Cambridge, MA) kindly provided the poxviruses. Expression of recombinant NeuT encoded by rV-*neuT* was detected by Western blotting after infection of BSC-1 cells with V-wt or rV-*neuT*. Cells were infected with 10 PFU/cell of poxviruses and cultured at 37°C for 18 h. Cell lysates, protein concentrations and immunoblotting were conducted as previously described [33, 36]. Polyclonal anti-ErbB2/neu antiserum was used to detect recombinant NeuT.

Transgenic BALB-*neuT* mouse colony

Transgenic BALB-*neuT* male mice were routinely mated with BALB/c females (H-2^d; Charles River, Calco, Italy) in the animal facilities of Tor Vergata University. Progenies were confirmed for presence of the transgene by PCR [27]. Individually tagged virgin females were used in this study. Mice were bred under pathogen-free conditions and handled in compliance with European Union and institutional standards for animal research.

Recombinant vaccinia neu vaccination

Protocol of vaccination was approved by the Ethical Committee of the University of Rome “Tor Vergata” and submitted to the Italian Health Department. Groups of BALB-*neuT* mice were vaccinated by s.c or im.g injection. Subcutaneous injection was performed at the base of the tail. Viruses were diluted in PBS (Phosphate Buffered Saline) such that the entire dose was delivered in 100 µl. Mice received for each vaccination a total of 10⁸ pfu (plaque forming unit) of either rV-*neuT* or V-wt. For intramammary gland delivery, mice received 10⁷ pfu in each mammary gland for a total of 10⁸ pfu each dose. BALB-*neuT* mice were vaccinated starting at the age at which they displayed atypical breast hyperplasia (6 weeks). One group of mice was vaccinated only one time (1X), while other groups were vaccinated once and then boosted one (2X) or two times (3X) at 4-week intervals (complete schedules: 6, 10 or 6, 10, 14 weeks). Other groups of BALB-*neuT* mice were vaccinated one time with rV-*neuT* or V-wt starting at the age coinciding with *in situ* (11 weeks) or invasive breast carcinoma (16 weeks). Groups of mice were boosted one or two more times every 4 weeks (complete schedule: 11, 15, and 11, 15, 19 or 16, 20, and 16, 20, 24 weeks, respectively). Depending on the immunogen, groups of 5–17 mice were vaccinated. The number of BALB-*neuT* mice receiving one (1X) or two (2X) doses of rV-*neuT* or V-wt were 5 in each group, independently of the time of initial vaccination and type of delivery. The number of

mice receiving three doses (3X) of s.c rV-*neuT* or V-wt were 10, 17, 12 and 8, 13, 10 when the vaccination was started at 6, 11 and 16 weeks of age, respectively. The number of mice receiving 3X of im.g rV-*neuT* or V-wt were 9, 13, 16 and 7, 7, 13 when the vaccination was started at 6, 11 and 16 weeks of age, respectively.

Analysis of anti-tumor activity *in vivo*

Mammary glands were checked weekly and tumors recorded at 3 mm in diameter. Tumor growth was monitored until all mammary glands displayed a palpable tumor or tumor mass exceeded 15 mm in diameter. At this point mice were sacrificed. Time of initial tumor appearance as well as tumor multiplicity were averaged as the mean $\pm$ standard deviation of incident tumors [33].

Antibody immunity following vaccination with rV-*neuT*

Sera from vaccinated BALB-*neuT* mice were collected prior to vaccination and 7 days after the final boost. Serum from animals vaccinated one time was collected 4 weeks after the vaccination. The presence of antibodies reactive to Neu was assayed using NIH3T3, LTR-Neu and TUBO cells by immunoblotting, immunofluorescence or ELISA (Enzyme Linked Immunosorbent Assay) as previously described [33, 36]. For immunofluorescence, mouse serum was used at 1:2000 [33]. For ELISA, individual rV-*neuT* mouse serum at different dilutions (1:500, 1:4000, 1:16000) was assayed against LTR-Neu and NIH3T3 control (5×10^4 cells/well). The specific absorbance of each sample was calculated by subtracting LTR-Neu absorbance from that of NIH3T3 cells. Antibody titer was estimated as the highest immune serum dilution generating a specific absorbance of 2.2 at 492 nm. Sera titer is the mean value of individual serum titer [37]. Individual serum samples from mice receiving 3X of rV-*neuT* at 11 (n=12) and 16 (n=10) weeks were randomly chosen. Individual V-wt mouse serum was assayed at 1:250 dilution. Immunoglobulins subclasses were determined by ELISA using a Mouse Typer Isotyping Kit (Biorad, Richmond, CA, USA) using individual serum of rV-*neuT* vaccinated mice as previously described [37, 38].

Biologic activity of vaccinated mouse immune sera *in vitro*

Antibody dependent cellular cytotoxicity (ADCC) was conducted as previously described [32, 33]. BALB-*neuT* mammary tumor cells (5×10^3 cells/well) were used as targets (T), while spleen cells from normal BALB/c mice were used as effectors at 50:1. Dilutions of sera pooled from four mice vaccinated 3X with rV-*neuT* or V-wt starting at the age of 6 and 16 weeks were assayed. Dilutions of sera from s.c (1:10, 1:20, 1:40) or im.g (1:12, 1:24, 1:48 and 1:11, 1:22, 1:44 for mice vaccinated at 6 and 16 weeks, respectively) rV-*neuT* vaccinated mice were normalized according to their magnitude of reactivity to Neu as determined by ELISA. Percentage of specific lysis was calculated as described [32, 33]. Results represent average percent cytotoxicity of three independent experiments. Four randomly chosen serum samples were pooled and used for two independent experiments. Four other randomly chosen serum samples were pooled and used for the third experiment. For *in situ* detection of programmed cell death of BALB-*neuT* cancer cells, immunoglobulins (Ig) from BALB-*neuT* mice pooled sera were purified by protein G and dialyzed against PBS. BALB-*neuT* cancer cells (2.5×10^3 cells/well) were incubated in serum-free DMEM containing 0.2% BSA containing Ig (10 μ g/ml) from rV-*neuT* or V-wt vaccinated mice starting at the age of 6 weeks. Ig were replenished each 24 hours. Cells were fixed in 4% formaldehyde for 15 min and after washing they were incubated with the polyclonal anti-activated caspase-3 antibody for 1 hour. After another washing cells were labeled with goat anti-rabbit IgG Alexa fluor-594-conjugated antibody (Invitrogen) for 30 min [36]. After a third washing cells were incubated with 0.1 μ g/ml Hoechst 33342 and mounted under a coverslip in glycerol. Staurosporine at 1 μ M for 24 h was used as positive control. The percentage of apoptotic cells was calculated by determining the activated

caspace-3 positive cells/total cells evaluating five randomly chosen microscopic fields. Count of apoptotic cells was done in a blinded fashion.

Detection of apoptotic cells *in vivo*

Mammary tissue from 2–3 BALB-*neuT* mice vaccinated with rV-*neuT* or V-wt starting at the age (6 weeks) coinciding with atypical hyperplasia was processed for immunohistochemical analyses as previously described [33, 36]. Three tumors were used for each group of vaccinated mice. Deparaffined tissue sections were incubated with the anti-activated caspase-3 antibody [39]. Apoptotic cells were counted at 200X in 5 microscopic randomly chosen fields.. This result represents the mean $\pm$ standard deviation of positive cells/field evaluated by immunohistochemistry. Count of apoptotic cells was done in a blinded fashion. Electron microscopy analysis was performed as previously described [40]

IL-2 and IFN- γ release assay

Spleen cells from BALB-*neuT* vaccinated mice at 16 weeks of age were harvested 7 days after the final vaccination as previously described [33]. Spleen mononuclear cells (2×10^6 /well in 24-well plates) were incubated with Concanavalin A (ConA, 2 μ g/ml), various Neu peptides (10 μ g/ml) or control gag peptide. Neu peptides were selected based on immunoreactivity *in vitro* with lymphocytes from BALB-*neuT* mice vaccinated with recombinant adenovirus or NIH3T3 fibroblasts (LTR-Neu) expressing Neu [30, 33]. IL-2 and IFN- γ release into the supernatant was measured using an enzymatic immunocapture assay (Quantikine®, R&D Systems, Minneapolis MN). Results represent the mean of two independent experiments.

Statistical analysis

Mean and standard deviation describe continuous variables. Survival curves were estimated using the Kaplan-Meier method and compared by the log-rank test. The effects of vaccine, route of administration, number of injections and starting point of the vaccination on time to initial tumor appearance were estimated using the Cox proportional hazards model. Ties in the failure times were handled by computing the exact conditional probability, under the proportional hazards assumption, that all tied event times occur before censored times of the same value or before larger values. Diagnostics based on the weighted Schoenfeld residuals did not show any significant departure from the proportional hazards assumption. The same factors were considered as potential predictors of reduction of tumor multiplicity at 25 weeks; their effects were estimated by the Poisson model, introducing an additional parameter to adjust for over-dispersion. Differences in number of apoptotic cells, titer of the serum, isotype of immunoglobulins and percent of antibody-dependent cellular cytotoxicity were evaluated by a two-tailed t-test. A preliminary test was performed to compare variances between groups; if a significant difference was detected, the classical t-test statistics were modified and the Satterthwaite's approximation utilized.

Results

Expression of recombinant NeuT encoded by rV-*neuT*

Expression of recombinant NeuT encoded by rV-*neuT* was detected by Western blotting after infection of BSC-1 with rV-*neuT*. Polyclonal anti-HER-2/neu antibody detected a 185 kDa protein product on BSC-1 cells infected with rV-*neuT* but not in those infected with the wild type virus, V-wt (data not shown).

Inhibition of mammary carcinogenesis by recombinant vaccinia neu (rV-*neuT*) vaccine: effect of stage of initial vaccination, multiple injections and route of delivery

To compare the effectiveness of systemic vs local route of rV-*neuT* administration, we employed s.c or im.g vaccination, respectively. To determine whether efficacy of vaccination was dependent on the carcinogenesis stage at which the vaccination was initiated, BALB-*neuT* mice were initially vaccinated at the stage of atypical hyperplasia (6 weeks), carcinoma in situ (11 weeks) and invasive carcinoma (16 weeks). In addition, to determine the usefulness of multiple rV-*neuT* injections, a dose response study (1X, 2X and 3X) was carried out. Control groups of mice received wild-type vaccinia virus (V-wt). Results by Cox's model show that all of the four considered variables (i.e., V-wt vs rV-*neuT* vaccination, multiple injections, route of delivery and disease stage of initial vaccination) significantly affect the tumor-free survival (Table 1). Overall, our results indicated specific interference of the rV-*neuT* vaccine with tumor growth and dependency of the effect on the number of injections, starting point and modality of the vaccination (Table 1). When considering the effectiveness of the rV-*neuT* vaccine independently of the number of injections, route and beginning of vaccinations, the estimated tumor-free survival of mice vaccinated with rV-*neuT* vs those receiving the V-wt was 0.24 (s.d. 0.037) vs 0 at 40 weeks, respectively, and the estimated median tumor-free survival time was 31 vs 20 weeks (Figure 1, panel A). Overall, at 30 weeks of age, the estimated tumor-free survival of mice vaccinated with one, two or three rV-*neuT* doses was 0.1 (s.d 0.055), 0.5 (s.d. 0.091) and 0.7 (s.d 0.299), respectively, with an estimated median tumor-free survival time of 26, 30.5 and 36 weeks (Figure 1, panel B). The dose dependent response was observed independently of the time in which the rV-*neuT* vaccination was initiated and route of injection (Table 1). Thus, our results indicate that the regimen protocol using three rV-*neuT* injections is the most effective in inducing anti-tumor activity (Table 1). In addition, our results indicate that early vaccination (6 and 11 weeks of age) improves the antitumor effectiveness of the rV-*neuT* vaccine (Table 1) (Figure 1, panel C). Reduction of tumor multiplicity at 25 weeks was also dependent on the starting point and number of vaccinations (Supplementary Table S1).

Overall, the risk of developing tumors in the im.g rV-*neuT* vaccinated group was 0.529 in comparison to the s.c vaccinated group (Table 1). For example, the Kaplan-Meier method showed that when rV-*neuT* vaccination was started at 16 weeks, three vaccinations provided an estimated median tumor-free survival time of 28.5 vs 25 weeks, respectively, for im.g vs the s.c rV-*neuT* vaccination (Figure 1, panel D). At this stage of vaccination the estimated tumor-free survival at 30 weeks was 0.375 (sd=0.121) vs 0.25 (sd=0.125) when three vaccinations were performed by im.g vs s.c rV-*neuT* injection, respectively (Figure 1, panel D). Differences in tumor-free survival between s.c or im.g rV-*neuT* 3X vaccinated mice when the vaccination was started at 6 and 11 weeks of age are also showed in Figure 1, panel D. In addition, at 25 weeks of age, tumor multiplicity was significantly different between s.c and im.g vaccination (Supplementary Table S1).

Our findings indicate that im.g rV-*neuT* vaccination is superior to s.c vaccination in inhibiting the neu oncogene-mediated mammary carcinogenesis.

Anti-Neu humoral response following rV-*neuT* vaccination

Previous studies demonstrated that a potent anti-Neu humoral response is necessary to prevent mammary tumor growth in BALB-*neuT* vaccinated mice [32]. To determine whether differences in humoral response exist between multiple rV-*neuT* injections or the carcinogenesis stage at which the rV-*neuT* vaccination was initiated and between rV-*neuT* s.c and im.g route of administration, specific antibody response to Neu was quantitatively and qualitatively evaluated by ELISA, immunoprecipitation following Western blotting and immunofluorescence. Specific anti-Neu reactivity of rV-*neuT* vaccinated mouse serum was

visualized by immunoblotting of immunoprecipitates using the anti-HER-2/neu specific antibody and corroborated by immunofluorescence (Supplementary Figure S1, panel A and B). The magnitude of the immune response elicited by varying doses of rV-*neuT* and the most effective route of rV-*neuT* vaccination were quantitated by ELISA. As shown in Table 2, mice vaccinated 3X with rV-*neuT* by the s.c route developed a significantly higher titer of anti-Neu antibodies than those vaccinated 1X or 2X, independently of the starting point of vaccination. Similar results were obtained when mice received one, two or three im.g rV-*neuT* doses. A comparison between the two routes of administration clearly showed that the im.g vaccination was more effective in eliciting anti-Neu antibodies than the s.c vaccination when two or three vaccinations were performed independently of the starting point of the vaccination (Table 2). Furthermore, when the BALB-*neuT* vaccination started at the stage in which the mice displayed atypical breast hyperplasia, even the administration of 1X im.g rV-*neuT* resulted in antibody titers higher than those observed with 1X s.c rV-*neuT*. The administration of V-wt did not result in the induction of anti-Neu antibodies.

Experiments were then carried out to evaluate the isotype of the immunoglobulins elicited by rV-*neuT* vaccination. For comparison, sera of BALB-*neuT* mice vaccinated 1X or 3X with rV-*neuT* by the s.c or im.g routes were analyzed (Table 3). As shown in Table 3, after three vaccinations the population of IgM significantly decreased in mice vaccinated three times compared to those receiving only one dose of rV-*neuT* independently of the route of vaccination. However, when one or three doses were given beginning at 6 or 16 weeks of age, the im.g route of vaccination resulted in significant enhancement of the IgG2a population compared with the s.c vaccination. Furthermore, after three vaccinations, a significant increase of the IgG3 population was observed in those animals receiving rV-*neuT* by the im.g route as compared to those vaccinated by the s.c route. The increase of the IgG2a/G3 populations was paralleled in the im.g vaccinated mice by the decrease of the IgG1 population. These results indicated that the rV-*neuT* route of vaccination affects the anti-Neu immunoglobulin specific isotype.

Biological activity *in vitro* of immune sera of rV-*neuT* vaccinated mice

Differences in IgG populations induced by the s.c and im.g routes of vaccination might possibly mirror dissimilarities in biological activity of immune sera of rV-*neuT* vaccinated mice. To test this hypothesis, antibody-dependent cellular cytotoxicity (ADCC) of BALB-*neuT* mammary tumor cells (TUBO) was analyzed using sera of mice vaccinated at 6 and 16 weeks of age (Figure 2, panel A). Spleen cells produced no cytotoxicity in the presence of the sera of V-wt vaccinated mice. Conversely, spleen cells in the presence of sera of s.c or im.g rV-*neuT* vaccinated mice showed a high degree of cytotoxicity. However, spleen cells stimulated with the im.g rV-*neuT* serum at 1:10 and 1:20 dilutions were more effective in their ability to elicit ADCC than those in the presence of the s.c rV-*neuT* serum both for sera of mice vaccinated at 6 and 16 weeks of age (Figure 2, panel A).

To determine whether specific immunoglobulins were able to trigger apoptosis, BALB-*neuT* tumor cells were labeled with anti-activated caspase-3 polyclonal antibody upon chronic treatment with Ig (10 µg/ml) from BALB-*neuT* mice vaccinated with rV-*neuT* or V-wt starting at the age of 6 weeks. Figure 2, panel B shows detection of cleaved caspase-3 in BALB-*neuT* cells. The fraction of apoptotic cells was determined relative to cleaved-caspase 3 positive cells. Purified Ig from s.c or im.g rV-*neuT*-vaccinated mice induced apoptosis of 41.7% and 55.5%, respectively ($p=0.0007$). In comparison, treatment with Ig from V-wt vaccinated mice triggered irrelevant BALB-*neuT* cell apoptosis (0.6 % and 1.5% for the s.c or im.g route, respectively). Treatment of cells with 1 µg/ml staurosporine resulted in 90% apoptotic cells.

Our results demonstrate that *in vitro* biologic activity including ADCC and induction of apoptosis by sera from mice vaccinated by s.c or im.g route corresponded to their differential ability to interfere with tumor growth *in vivo*.

T-cell immune response by rV-*neuT* vaccination

Studies were then undertaken to determine whether the different routes of rV-*neuT* administration elicit dissimilar Neu T cell immunity. Splenocytes isolated from mice vaccinated at 16 weeks of age after the third vaccination were examined for their ability to proliferate under various Neu peptides. Release of IL-2 and IFN- γ was measured in the supernatant to assess T cell immunoreactivity with specific Neu epitopes. Results are reported in Table 4. T cell proliferative response to ConA was similar for all the vaccinated groups. All Neu peptides analyzed, but not an unrelated gag peptide, were able to specifically activate splenocytes from BALB-*neuT* mice vaccinated with rV-*neuT*. However, the magnitude of IL-2 and IFN- γ secretion was conditioned by the stimulating Neu peptide. The strongest T cell response was observed for both groups of rV-*neuT* vaccinated mice upon stimulation with the 166, 156, 141 and 15.3 peptides, the first located in the transmembrane domain while the remaining were in the extracellular domains of rat Neu sequence. A weaker IL-2 and IFN- γ release was detected upon stimulation with other Neu peptides located in the extracellular domain (r41 and r98). No significant divergence for specific recognition of Neu peptides was observed between splenocytes from s.c or im.g rV-*neuT* vaccinated mice. T cells from im.g rV-*neuT* vaccinated mice release in general higher IL-2 and IFN- γ than those from s.c rV-*neuT* mice upon stimulation with the 166, 156 and 141 Neu peptides. However, these differences were not significant (Table 4).

In vivo induction of apoptosis in mammary tumors following rV-*neuT* vaccination

To determine whether rV-*neuT* vaccination of BALB-*neuT* mice induces *in vivo* mammary cancer cell apoptosis, tumor breast tissues from rV-*neuT* or V-wt vaccinated mice by s.c or im.g route, starting at the age coinciding with atypical hyperplasia, were analyzed by immunohistochemistry for expression of activated caspase-3 on cancer cells. Figure 2, panel C shows detection of apoptotic cancer cells in mammary tissue. Activated caspase-3 positive cancer cells were counted in microscopic randomly chosen fields. Mammary tumors from V-wt vaccinated mice showed a very small number of apoptotic cancer cells (0.2 ± 0.5 and 0.3 ± 0.5 for the s.c and im.g vaccination, respectively). Conversely, vaccination with rV-*neuT* was associated with a noticeable number of apoptotic cancer cells detected among areas of ischemic and hemorrhagic necrosis in mammary tumors. Mammary tissue from rV-*neuT* vaccinated mice displayed 7.1 ± 2.2 and 12 ± 3.5 apoptotic cancer cells per field when the s.c and im.g routes of vaccination were employed, respectively. Differences in the number of cancer apoptotic cells were significant between rV-*neuT* and V-wt vaccination (1×10^{-7} and 1×10^{-8} for s.c and im.g vaccination, respectively) as well as between rV-*neuT* s.c and im.g vaccination ($p=0.005$). This latter evidence further confirms differences in anti-tumor activity elicited by im.g vs s.c route of vaccination. The presence of apoptotic cancer cells within necrotic areas and tumor infiltrating lymphocytes has also been demonstrated by ultrastructural analysis in mammary tissue from rV-*neuT* im.g vaccinated mice (Supplementary Figure S2).

Discussion

Clinical studies have demonstrated the ability of trastuzumab, a recombinant humanized monoclonal antibody, which recognizes the extracellular domain of the ErbB2 protein to induce an objective response in breast cancer patients [41, 42]. These studies, however, have also revealed that the objective response to trastuzumab monotherapy had a median duration of 9 months, and that the majority of responsive patients displayed resistance within 1 year

[42]. Conversely, it has been also demonstrated that combination therapy with trastuzumab and a HER2/neu vaccine was associated with minimal toxicity and results in prolonged, robust, antigen-specific immune responses in treated patients [43]. In light of these findings it is realistic to explore ErbB2 cancer vaccine approaches with the aim to improve the objective tumor inhibitory response [44]. Active vaccination using ErbB2 as immunogen might maintain tumor inhibition more effectively than passive immunotherapy based on the induction of a persistent memory immune response and induction of T and B cell immunity to multiple immunodominant epitopes. However, there are safety concerns in vaccination involving a potent oncogene like ErbB2. Since oncogenic ErbB2 function relies on its intrinsic tyrosine kinase activity, elimination by mutation of its kinase domain represents a feasible alternative. Mice transgenic for the rat neu oncogene (Balb-*neuT*) are used to evaluate the capacity of ErbB2/neu vaccines to inhibit the progression of neu-driven carcinogenesis [28]. Recombinant vaccinia virus encoding for tumor associated antigens has been widely employed in phase I clinical trials for the treatment of advance stage cancer patients [6–11, 13, 14, 17, 19].

Although these trials proved the safety of vaccinia virus vaccination, as well as T and B cell responses to the encoded tumor associated antigen in some cases, they showed only a small degree of clinical benefit for cancer patients [6–11, 13, 14, 17, 19]. Poxvirus represents an attractive delivery vehicle of tumor antigens due to the normal post-translational modification of the inserted antigen and strong immunogenicity [3–5]. In this study we explored for the first time the mammary tumor inhibitory ability of the recombinant vaccinia virus neu (rV-*neuT*) vaccine when administered in BALB-*neuT* mice. We also set out to determine whether the vaccination efficiency of rV-*neuT* vaccine was dependent on the carcinogenesis stage at which the vaccination was initiated and on the use of multiple rV-*neuT* injections.

Our observations indicated that tumor suppression was more effective when started at an earlier stage of the disease. The degree of tumor growth interference *in vivo* reflected the titers of anti-Neu serum antibodies elicited upon rV-*neuT* vaccination. Regression of established tumors following vaccination was less effective, most likely due to insufficient antibody accessibility or higher antibody requirement *in vivo*. In addition, we showed that immune response and antitumor activity were increased by repeated rV-*neuT* vaccinations. However, one of the potential drawbacks in the use of multiple recombinant vaccinia administrations is that preexisting and/or induced antibody and T cell-response to vaccinia virus will prevent the spread of the inoculated vaccinia virus and thus diminish the expression of inserted antigen. On the other hand, it should be noted that smallpox was eradicated worldwide more than 25 years ago; thus young women are no longer vaccinated. In addition, recombinant avipox virus, which has a limited viral replication, can be used to boost immune response after recombinant vaccinia priming [4]. It was previously demonstrated that the mechanism of tumor protection in BALB-*neuT* depends on the antibody-mediated blockade of Her-2/neu function [31, 32]. In the current study we provided evidence that immune sera from rV-*neuT*-vaccinated mice induced apoptosis of BALB-*neuT* tumor cells *in vitro*, which corresponded to the relative extent of tumor inhibitory effect *in vivo*. We also demonstrated that immune rV-*neuT* sera were able to mediate ADCC. Furthermore, we demonstrated *in vivo* induction of cancer cell apoptosis in BALB-*neuT* mammary tumor sections following rV-*neuT* vaccination, by detecting activated caspase-3 positive cancer cells. This immunohistochemical finding was corroborated by the presence of apoptotic bodies within necrotic areas and tumor infiltrating lymphocytes in the tumor mass, as demonstrated by ultrastructural analysis.

Vaccination with recombinant vaccinia virus can be achieved by systemic or local intratumoral injection [3, 6–19]. Although the majority of anti-cancer vaccine strategies

employ systemic vaccination, recent data support the effectiveness of the intratumoral vaccination both in human and experimental models [11, 13, 15–25]. A prerequisite to employing intratumoral vaccine therapy is the access to the tumor site. The accessibility of breast tumors and the standard surgical removal of the tumor allow one to envision intratumoral immunotherapy in a neoadjuvant setting [22].

In the current study we compared the antitumor effect induced by systemic vs local route of rV-*neuT* administration, by employing subcutaneous (s.c) vs intramammary gland (im.g), respectively. Our findings indicate that rV-*neuT* im.g vaccination is superior to s.c vaccination in inhibiting the neu oncogene-mediated mammary carcinogenesis. Furthermore, we demonstrated that rV-*neuT* im.g vaccination was more effective in eliciting anti-Neu antibodies and in increasing anti-Neu IgG2a/G3 isotypes than s.c vaccination. Immunoglobulins of the IgG2a isotype have been shown to mediate in mice a more potent ADCC than other Ig isotypes [45]. The *in vitro* biologic activity including ADCC and induction of cancer cell apoptosis by sera from im.g vaccinated mice was superior to that induced by sera from s.c vaccinated mice. It has been demonstrated that cytokines release and antibody production are the immune mechanisms mostly responsible for tumor protection in BALB-*neuT* mice, whereas cytotoxic T lymphocytes appear to play a marginal role [32, 46]. In addition, cytotoxic T cells reacting with high affinity with rat Neu are deleted in BALB-*neuT* mice by central tolerance [47]. Here, we showed that T cells from s.c and im.g rV-*neuT* vaccinated mice release IL-2 and IFN- γ upon stimulation with the 166, 156, 141 and 15.3 peptides, the first located in the transmembrane domain, while the remaining are in the extracellular domains of rat neu sequence. However, we could not detect significant differences in response to Neu peptides between splenocytes from s.c or im.g rV-*neuT* vaccinated mice.

The differential level of humoral immune response between the s.c and im.g routes of vaccination paralleled their differential ability to interfere with tumor growth *in vivo*. A superior degree of *in vivo* induction of mammary cancer cell apoptosis in rV-*neuT* im.g vaccinated mice further supports this finding. Poxvirus infection leads to the production of immunomodulatory proteins that activate the innate immune system, a crucial event to induce a strong adaptive immune response. Such immunomodulatory proteins include interferons, chemokines, inflammatory cytokines, and the Toll-like receptor family of pattern recognition receptors [2]. According to the “danger” model proposed by Matzinger, the immune system is activated by danger signals from injured tissues so that any molecule independently, whether foreign or self, can induce a specific immune response if it is able to alert and activate a specialized APC which in turn expresses costimulatory molecules and promotes T and B cell activation [48, 49]. Local vaccination with recombinant vaccinia virus might provide danger signals more proficiently than systemic vaccination. As a matter of fact, BALB-*neuT* V-wt im.g vaccinated mice had a detectable superior tumor-free survival than those vaccinated by s.c vaccination. Furthermore, the combination of a neu genetic vaccine and novel agonist of TLR9 had potent antitumor activity associated with antibody isotype switch and antibody-dependent cellular cytotoxicity activities. Mice treated with the combination produced greater antibody titers with IgG2a isotype switch and antibody-dependent cellular cytotoxicity activity than did mice treated with the vaccine alone [50]. It was also reported that intratumoral delivery of CpG has advantages in the treatment of tumors [51]. Rituximab, a chimeric monoclonal antibody against the protein CD20, plus intratumoral CpG could eradicate B cell lymphoma from 42% of mice, whereas systemically administered CpG, with or without rituximab, did not achieve tumor eradication [52].

Our findings may have important implications for the design of cancer vaccine protocols for the treatment of breast cancer and other accessible tumors using recombinant vaccinia virus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Inhibition of neu oncogene-mediated mammary carcinogenesis *in vivo* by rV-*neuT* vaccination

(A) Differences in tumor-free survival between V-wt and rV-*neuT* vaccinated BALB-*neuT* mice independently of dose, starting point and route of vaccination, (B) Differences in tumor-free survival between V-wt and rV-*neuT* vaccinated BALB-*neuT* mice after multiple injections, (C) Differences in tumor-free survival between V-wt and rV-*neuT* vaccinated BALB-*neuT* mice when the vaccination was started at 6, 11 and 16 weeks of age, (D) Differences in tumor-free survival between s.c or im.g rV-*neuT* 3X vaccinated mice when the vaccination was started at 6, 11 and 16 weeks of age. Number of vaccinated mice is reported in Materials and Methods section.

Figure 2. Biological activity *in vitro* of immune sera or purified immunoglobulins of rV-neuT vaccinated mice and *in vivo* detection of apoptosis induced by rV-neuT vaccination

(A) Biological activity *in vitro* of sera from rV-neuT vaccinated mice. Specific antibody-dependent cell-mediated cytotoxicity was elicited by rV-neuT sera from mice vaccinated at 6 and 16 weeks of age. BALB-neuT mammary cancer cells were exposed for 2 h to sera pooled from s.c or im.g rV-neuT or V-wt vaccinated mice at different dilutions (for s.c, 1=1:10, 2=1:20, 3=1:40 and for im.g, 1=1:12, 2=1:24, 3=1:48 (mice vaccinated at 6 weeks) and 1=1:11, 2=1:22, 3=1:44 (mice vaccinated at 16 weeks). Results represent average percent cytotoxicity of three independent experiments. *=0.0287, **=0.0362, ***=0.033, ****=0.046: p-values for im.g vs s.c rV-neuT 3X vaccination, (B) Induction of apoptosis by rV-neuT immunoglobulins in BALB-neuT mammary tumor cells. Apoptotic cells were identified when positively stained by the anti-activated-caspase 3 polyclonal antibody. Immunoglobulins from vaccinated mice starting at the age of 6 weeks: a) s.c V-wt, b) im.g V-wt, c) negative control, d) s.c rV-neuT, e) im.g rV-neuT, f) staurosporine treated cells. Nuclei were counterstained with Hoechst. Original magnification $\times 200$, (C) Immunohistochemical analysis detecting apoptotic cells by anti-activated-caspase3 polyclonal antibody in mammary tumors developed in s.c and im.g rV-neuT or V-wt vaccinated BALB-neuT mice starting at the age coinciding with atypical hyperplasia (6 weeks). Immunoperoxidase counterstained with hematoxylin. Original magnification $\times 200$.

Multivariate analysis of tumor-free survival of BALB-*neuT* mice after rV-*neuT* vaccination according to the Cox model

Table 1

Variable	Contrast	Hazard ratio	95% hazard ratio confidence limits	p-value
Vaccine	rV- <i>neuT</i> vs V-wt	0.007	0.003	<0.0001
Route of administration	im.g vs s.c	0.529	0.396	<0.0001
Number of injections	2 vs 1	0.363	0.245	<0.0001
	3 vs 2	0.333	0.214	<0.0001
Starting point of the vaccination(weeks)	11 vs 6	1.857	1.290	0.0009
	16 vs 11	3.256	2.131	<0.0001

Table 2

Immunoreactivity of rV-*neuT* vaccinated Balb-*neuT* mouse sera with Neu.

Starting point and dose (Number of mice with immune response/total) ^a	Type of delivery of rV- <i>neuT</i>	Number of pooled mouse sera	Serum Titer Mean (Sd) ^b	im-g vs s.c p value (t-test)	2X vs 1X*3X vs 2X ♦ p value (t-test)
6 weeks					
1X (5/5)	s.c	5	1295 ^c (72)		
1X (5/5)	im-g	5	2328 (213)	<0.0001	
2X (5/5)	s.c	5	2270 (441)		0.0002*
2X (5/5)	im-g	5	3720 (164)	0.0001	0.0335*
3X (10/10)	s.c	10	8660 (207)		<0.0001 ♦
3X (9/9)	im-g	9	10800 (1565)	0.0371	<0.0001 ♦
11 weeks					
1X (5/5)	s.c	5	1780 (76)		
1X (5/5)	im-g	5	2460 (657)	0.0813	
2X (5/5)	s.c	5	2705 (273)		0.0351 *
2X (5/5)	im-g	5	3320 (45)	0.0067	0.0391 *
3X (17/17)	s.c	12	11083 (970)		<0.0001 ♦
3X (13/13)	im-g	12	13667 (753)	0.0004	<0.0001 ♦
1X (5/5)	s.c	5	1065 (251)		
1X (5/5)	im-g	5	1390 (342)	0.1249	
2X (5/5)	s.c	5	2710 (286)		<0.0001 *
2X (5/5)	im-g	5	3620 (533)	0.0099	<0.0001 *
3X (12/12)	s.c	10	12880 (164)		<0.0001 ♦
3X (16/16)	im-g	10	14006 (583)	0.0104	<0.0001 ♦

^a Immune response was determined by ELISA against LTR-Neu and NIH3T3 at 1:500 serum dilution. Specific absorbance for all rV-*neuT* sera was >1.0. These values were considered positive as compared to that obtained with V-wt sera. Optical density of V-wt sera (1X, 2X and 3X, 5 to 13 mice in each group) at 1:250 to LTR-Neu was <0.3. These values were considered negative;

^b Immune sera titers of BALB-*neuT* vaccinated mice were determined by ELISA against LTR-Neu and NIH3T3 using individual serum at different dilutions. Sera titer represents the mean value of individual serum titer. sd=standard deviation;

^c Titer was estimated as the highest immune serum dilution generating a specific absorbance of 2.2 at 492nm.

Table 3

Effect of rV-neuT vaccination on Balb-neuT immunoglobulin isotype sera

Starting point of vaccination	Dose	Type of delivery	Immunoglobulin isotype against Neu					
			IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
6 weeks								
rV-neuT	1×	s.c.	19.5 ± 1.6 ^a	20.8 ± 2.4	22 ± 1.6	8 ± 1.2	22.3 ± 5.9	7.8 ± 1.6
	1×	im.g.	16.2 ± 2.4	25.8 ± 2	20.2 ± 2.2	10.5 ± 0.8	19 ± 1.1	8 ± 2.5
<i>p</i>				0.001 ^b		0.03 ^b		
16 weeks								
rV-neuT	1×	s.c.	18.6 ± 1.1	19.6 ± 1	23.7 ± 3.4	9.7 ± 1.9	20.9 ± 1.1	7.2 ± 3.3
	1×	im.g.	19.7 ± 1.3	24.2 ± 1.1	19.9 ± 1.1	12.3 ± 2.9	16.2 ± 2.5	7.5 ± 0.4
<i>p</i>				0.01 ^b				
6 weeks								
rV-neuT	3×	s.c.	25.9 ± 2.1	38 ± 2.4	11.9 ± 1.4	8.8 ± 1.6	8.8 ± 1.1	6.9 ± 0.6
	3×	im.g.	18.7 ± 2	45.1 ± 2	11 ± 0.9	12.9 ± 0.6	7.7 ± 0.5	4.4 ± 0.9
<i>p</i>			0.02	0.005 ^b		0.016 ^b		
16 weeks								
rV-neuT	3×	s.c.	28.1 ± 0.7	36.5 ± 1.3	12.9 ± 0.5	8.6 ± 0.5	8 ± 1.1	5.7 ± 1.4
	3×	im.g.	19.8 ± 1.7	44.1 ± 0.7	12.5 ± 1.5	12.4 ± 1.2	6.8 ± 1.3	4.1 ± 0.6
<i>p</i>			0.005 ^b	0.002 ^b		0.024 ^b		

^a Results are the mean of the percent (±standard deviation) of each immunoglobulin isotype relative to the total sera immunoglobulin content (at 1:1,500).

^b im.g. versus s.c.

Table 4

T cell immune response of BALB-neuT mice following vaccination with rV-neuT

T cell in vitro stimulus	Neu peptide sequence	IL-2 release				IFN- γ			
		rV-neuT s.c.	V-wt s.c.	rV-neuT im.g.	V-wt im.g.	rV-neuT	V-wt s.c.	rV-neuT im.g.	V-wt im.g.
r15.3	TYVPANASL	200	4	198	11	205	4	209	4
r41	DMVLWKDVFRKNNQL	177	3	145	7	30	1	46	3
r98	IAPLRPEQLQVFETL	179	2	161	4	53	2	35	4
r141	LPCHPECPQNSSET	209	9	267	7	191	2	232	6
r156	GICQPCPINCTHSCV	325	13	417	15	196	2	219	6
r166	VLLFLILVVVVGILI	436	13	536	8	143	3	183	3
GAG		19	4	17	1	3	2	2	4
ConA		1,784	1,869	1,621	1,696	1,071	1,021	1,161	956

^a Spleen cells from vaccinated mice were stimulated in vitro with Neu-specific peptides. IL-2 and IFN- γ were quantitated in the supernatant (pg/ml) as a measure of T cell immunoreactivity with specific Neu epitopes. Concanavalin A (ConA) for global T cell activation and an unrelated gag peptide served as positive and negative control, respectively.