



## RESEARCH ARTICLE

# Insertion of the DNA for the 163–171 peptide of IL1 $\beta$ enables a DNA vaccine encoding p185<sup>neu</sup> to inhibit mammary carcinogenesis in Her-2/neu transgenic BALB/c mice

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An assessment was made of the effectiveness of DNA vaccination in prevention of the mammary adenocarcinomas of BALB/c female mice transgenic for the activated rat Her-2/neu oncogene. Atypical hyperplasia is evident in their mammary glands when they are 6 weeks old and in situ carcinoma by the 13th week. Palpable invasive carcinomas appear around the 17th week and are always evident in all 10 glands by the 33rd week. Intramuscular vaccinations with 100  $\mu$ g plasmid DNA encoding the extracellular domain of the Her-2/neu p185 (ECD) performed at the 6th, 12th, 18th and 24th week provided no significant protection, whereas those ECD plasmids in which the DNA coding for the

immunomodulatory 163–171 (VQGEESNDK) nonapeptide of human IL1 $\beta$  (ECD-IL1 $\beta$ p) had been inserted both delayed carcinogenesis and reduced tumor multiplicity. This reduction was associated with a marked immune-inflammatory reaction and a conspicuous leukocyte infiltrate located in the stroma surrounding the hyperplastic mammary ductal-alveolar structures. It was also directly correlated with a high anti-p185<sup>neu</sup> antibody production and an immunoglobulin switch to IgG2a and IgA. No anti-p185<sup>neu</sup> cytotoxic response was found. No significant protection was obtained when the DNA coding for the non-active peptide 189–197 of IL1 $\beta$  was inserted. Gene Therapy (2001) 8, 447–452.

**Keywords:** IL-1 $\beta$ ; DNA immunization; mammary carcinogenesis; Her-2/neu; tumor prevention

## Introduction

DNA vaccines are molecularly defined reagents that are easy to construct and elicit long-lasting cellular and humoral immune responses to a variety of antigens. Clinical trials have shown that they are nontoxic and well tolerated, though the responses their vaccination induce are low and vary from one individual to another,<sup>1–3</sup> while their efficacy is often limited by low levels of gene and protein expression and the complex requirements for protein presentation and lymphocyte activation.<sup>4</sup>

Enhancement of the potency of DNA vaccines has been sought through the employment of costimulatory molecules and cytokines as adjuvants.<sup>5,6</sup> Vaccines encoding antigens fused with immunological molecules and cytokines elicit more effective responses<sup>6,7</sup> and the ability of cytokines to enhance the immune recognition of tumor antigens has been extensively exploited.<sup>7</sup> Cytokine-gene engineered tumor cells,<sup>8</sup> and DNA encoding fusion proteins between cytokines and tumor antigens<sup>9</sup> induce marked immune responses, even against poorly immunogenic tumors.

Interleukin 1 (IL-1) is a particularly effective adju-

vant,<sup>10</sup> but its potent pyrogenic and/or proinflammatory properties drastically limit its use. The nonapeptide sequence VQGEESNDK corresponding to the amino acid stretch between the positions 163–171 of human IL-1 $\beta$ , on the other hand, is free from these properties and retains the immunostimulatory capability of the entire molecule.<sup>11,12</sup> Its local administration, in fact, markedly increase the immunogenicity of poorly immunogenic tumors in syngeneic mice.<sup>12</sup> Insertion of the DNA sequence encoding this nonapeptide in recombinant antigens enhances their immunogenicity.<sup>13</sup> DNA vaccination with plasmids encoding a fusion protein between idiotypic determinants of B cell lymphomas and this peptide induced a protective immune response against a subsequent lymphoma challenge.<sup>14</sup>

In this paper, we compare the ability of DNA vaccination with plasmids coding for the extracellular domain of product of rat Her-2/neu (p185<sup>neu</sup>) alone (ECD) or fused with the DNA coding for this IL-1 $\beta$  peptide (ECD-IL1 $\beta$ p) to block the progression of Her-2/neu carcinogenesis in female BALB/c mice transgenic for the activated rat Her-2/neu oncogene under the control of the MMTV promoter (BALB-neuT).<sup>15</sup> All the mammary glands of these mice independently undergo a very aggressive carcinogenesis that mirrors some features of the formation of lobular carcinoma in women.<sup>16</sup> Vaccination with plasmids coding for ECD alone did not block this carcinogenesis, whereas vaccination with ECD-IL1 $\beta$ p was followed by a significant delay.

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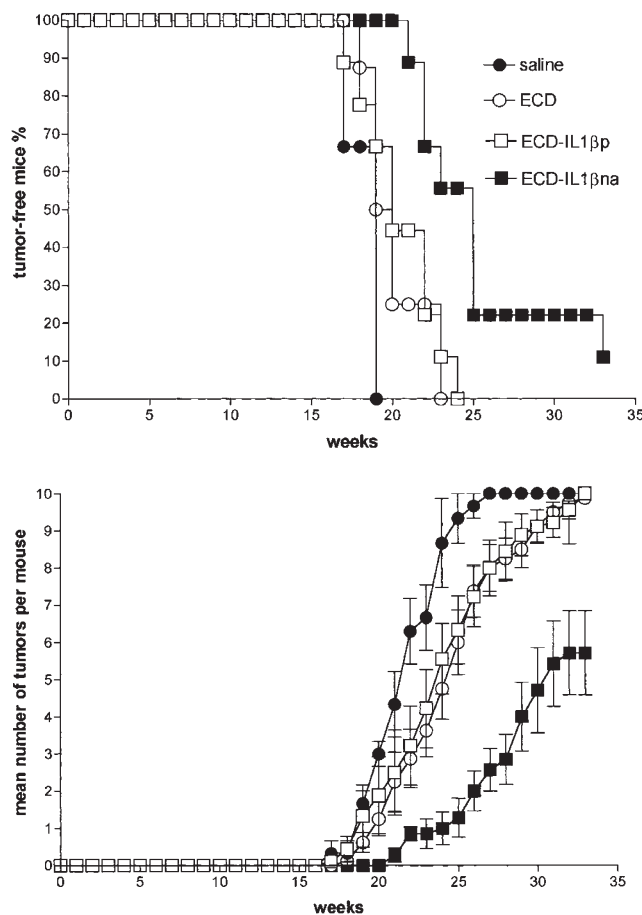
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## Results

### Immunization of BALB-neuT mice

Owing to expression of the activated rat Her-2/neu gene under the control of the MMTV promoter, high membrane overexpression of rat p185<sup>neu</sup> is already evident in the terminal ductular-lobular units of all the mammary glands of BALB-neuT female at the 3rd week of age. Atypical mammary hyperplasia is evident between week 3 and 6. Palpable invasive carcinomas appear around the 17th week in one or two glands of 40% of mice and are always evident in all 10 glands by the 33rd week.<sup>15,16</sup>

To assess the ability of DNA vaccination to hamper this progression, mice were immunized at the 6th, 12th, 18th and 24th week with plasmids coding for the ECD alone, ECD and IL-1 $\beta$ p (ECD-IL1 $\beta$ p), or ECD and the non-active peptide 189–197 of IL-1 $\beta$  (ECD-IL1 $\beta$ na). Vaccination with these plasmids did not hamper tumor growth, whereas immunization with ECD-IL1 $\beta$ p both delayed carcinogenesis and reduced tumor multiplicity (Figure 1). The mean number of mammary glands with palpable tumors at this time was six only in the ECD-IL1 $\beta$ p group, whereas all mice in the other groups displayed a palpable tumor in all their mammary glands (Figure 1, lower panel). More-

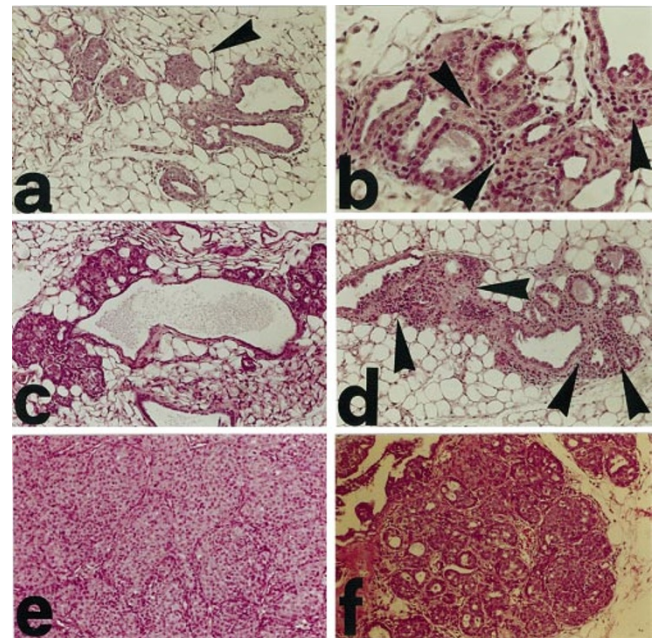


**Figure 1** Effect of vaccination with plasmids coding for ECD alone or fused with IL-1 $\beta$ p on the mammary carcinogenesis in BALB-neuT mice. The time of appearance of the first tumor (upper panel) and mean number of palpable mammary carcinomas per mouse (lower panel) in the group of eight mice injected with saline only (●), or vaccinated with ECD (○), ECD-IL1 $\beta$ na (□), and in a group of 10 mice immunized with ECD-IL1 $\beta$ p (■).

over, one of the 10 mice in the ECD-IL1 $\beta$ p group was still completely tumor free at week 33 (Figure 1, upper panel).

### Pathological analysis of mammary glands

Pathological observations performed at 7 weeks, 1 week after the first immunization showed that both mice injected with saline and immunized with ECD-IL1 $\beta$ p displayed foci of epithelial hyperplasia of the terminal ductular-lobular units (TDLU) (Figure 2). However, in immunized mice the TDLU were surrounded by a reactive leukocyte infiltrate (Figure 2b). At 13 weeks, 1 week after the second immunization mice injected with saline displayed a well-developed atypical epithelial hyperplasia. Epithelial cells were atypical and their growth inside the lumens distended and expanded the alveoli and lobules (Figure 2c). By contrast, mice immunized twice with ECD-IL1 $\beta$ p showed reduced hyperplasia and infiltrating reactive cells in the surrounding fibrotic stroma (Figure 2d). At 25 weeks, 1 week after the third immunization, a well-developed invasive lobular carcinoma was present in most mammary glands from control mice (Figure 2e), while only several foci of atypical hyperplasia and *in situ* carcinoma were found in some mammary glands of immunized mice (Figure 2f). These



**Figure 2** Histology of mammary tissue obtained from mice injected with saline (a, c, e) or immunized with ECD-IL1 $\beta$ p (b, d, f). At 7 weeks of age, mice injected with saline (a) show several foci of mammary ductulo-lobular hyperplasia constituted by a pluristratified epithelium sometimes occluding the alveolar lumen (arrowhead). By contrast the mammary tissue of immunized animals (b) shows that the ductulo-alveolar structures, mainly lined by a single epithelial cell layer, are clearly surrounded by lymphoid reactive cells (arrowheads). Thirteen-week-old mice injected with saline develop multifocal atypical hyperplasia (c) with several alveolar structures often filled by transformed epithelial cells. At the same age in immunized mice the hyperplasia is less pronounced and accompanied by an evident reactive cell infiltrate localized in the surrounding fibrotic stroma (arrowheads) (d). At 25 weeks of age, a well-developed invasive lobular carcinoma was present in several mammary glands of mice injected with saline (e) while, in those from immunized mice (f) hyperplasia with foci of *in situ* carcinoma bordered by reactive cell infiltrated fibrotic stroma was the prevalent pathological feature (a, c–f  $\times 200$ ; b  $\times 400$ ).

foci were bordered by a dense stroma markedly infiltrated by reactive leukocytes.

#### Cytotoxic response to p185<sup>neu</sup> positive target cells

The infiltrate and inhibition of the progression of carcinogenesis did not correlate with the induction of a detectable cytotoxic response in Spc collected 7 days after each vaccination and tested immediately or after 6 days *in vitro* restimulation with distinct p185<sup>neu</sup> target cells, as evaluated in 4 and 18 h <sup>51</sup>Cr release assays and 48 and 72 h [<sup>3</sup>H]TdR release assays (data not shown).

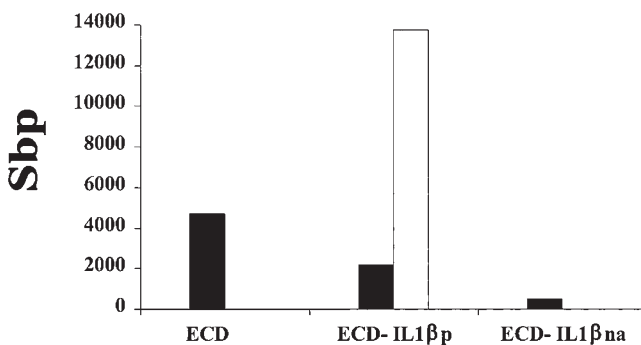
#### Antibody response associated with the inhibition of natural carcinogenesis

The ability of these treatments to induce anti-p185<sup>neu</sup> antibodies was evaluated in the sera collected at 33 weeks when all the mice vaccinated with saline, ECD or ECD-IL1βna displayed 10 large tumors. The antibody titer in mice with 10 large tumors was similar, irrespective of the vaccination with ECD, ECD-IL1βna or ECD-IL1βp. By contrast, the titer was much higher in the sera from mice vaccinated with ECD-IL1βp with only 1–4 tumors (Figure 3).

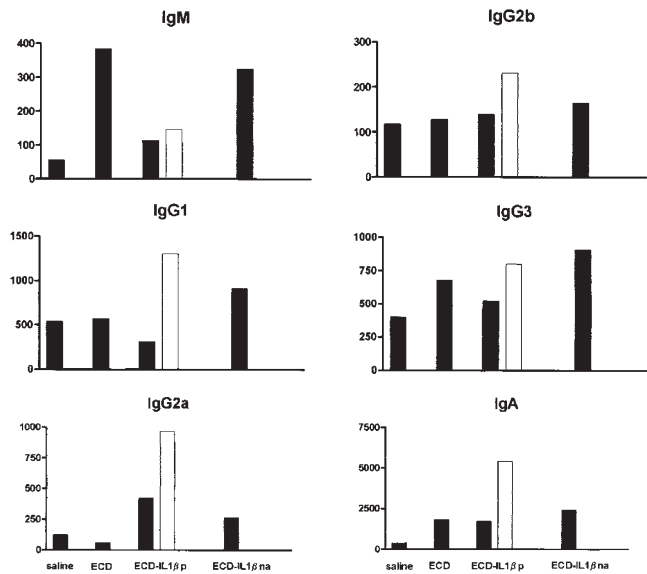
The distribution of immunoglobulin isotypes was also evaluated in these sera. IgM and IgG3 were increased in all immunized mice (Figure 4). In addition, mice with 1–4 tumors after ECD-IL1βp immunization presented an increase of several isotypes, especially IgG2a and IgA.

## Discussion

Present data show that insertion of IL-1βp DNA dramatically increases the protective efficacy of vaccination with plasmids coding for ECD. Only mice immunized with ECD-IL1βp plasmid display a delay in the appearance of the first tumor and a strong decrease of the number of mammary glands with a palpable carcinoma, whereas the natural consequence of activated rat Her-2/neu gene overexpression is that a large, fast-growing lobular carcinoma is palpable at week 33 in all 10 glands of control BALB-neuT mice injected with saline only. Neither vaccination with ECD nor with ECD-IL1βna plasmids significantly counteracts this aggressive carcinogenesis,



**Figure 3** Presence of anti-p185<sup>neu</sup> antibodies in the sera of variously immunized mice. Sera from mice immunized four times with ECD, ECD-IL1βp or ECD-IL1βna were collected at week 33, pooled and specific p185<sup>neu</sup> Sbp was evaluated by flow cytometry after indirect immunofluorescence. Black columns, sera from mice displaying a palpable tumor in all 10 mammary glands, irrespective of vaccination regimen. White column, serum pool from mice vaccinated with ECD-IL1βp displaying 1–4 mammary tumors only.



**Figure 4** Distribution of immunoglobulin isotypes in the sera of immunized BALB-neuT mice. Serum pools from mice injected four times with saline only or immunized with ECD, ECD-IL1βp or ECD-IL1βna tested by radial immunodiffusion. Black columns, sera from mice displaying a palpable tumor in all 10 mammary glands, irrespective of vaccination regimen. White columns, serum pool from mice vaccinated with ECD-IL1βp displaying 1–4 mammary tumors only.

whereas in a less aggressive model of Her-2/neu carcinogenesis, vaccination with the same ECD vector used in the present study provided a significant protection<sup>17</sup> that was further increased by coinjection of the ECD plasmid with a bicistronic vector coding for IL-12.<sup>18</sup>

This critical immunomodulatory role played by IL1βp fits in well with our previous experience using this peptide as a systemic adjuvant.<sup>12</sup> Moreover, its sustained local presence in the tumor area activates an effective antitumor reaction against a poorly immunogenic transplantable mammary tumor.<sup>12</sup> Insertion of IL1βp augments the antitumor immune response induced by protein and DNA vaccines.<sup>14</sup> Moreover, vaccination with plasmids containing IL1βp DNA sequence<sup>13,14</sup> or protein antigen fused with IL1βp<sup>13</sup> increases the immunogenicity of many antigens.

In BALB-neuT mice, rat p185<sup>neu</sup> is a self-protein already overexpressed by the mammary gland by the 3rd week of life.<sup>16</sup> Vaccination with ECD is unable to break this natural tolerance. Insertion of IL1βp DNA in the construct inhibits Her-2/neu carcinogenesis and is accompanied by lymphocyte infiltration of the stroma surrounding the TDLU and induction of anti-p185<sup>neu</sup> antibodies. By contrast, no significant CTL response was found, despite all the *in vitro* restimulation attempts. This provocative finding fits in well with the absence of cytotoxicity we have found following vaccination of BALB/c and BALB-neuT mice against plasmids encoding both the ECD and TM portions of the p185<sup>neu</sup>.<sup>19</sup> Even the presence of IL1βp signal is not enough to activate T killer cells against p185<sup>neu</sup>.

The distribution of anti-p185<sup>neu</sup> antibodies in the treatment groups also raises some puzzling issues. Only ECD-IL1βp immunized mice with evident inhibition of Her-2/neu carcinogenesis display a high anti-p185<sup>neu</sup> antibody titer. While this may suggest a direct correlation



between antibody titer and inhibition, the low titer found in mice with large tumors may be due to antibody absorption by the p185<sup>neu+</sup> tumor cells or immunosuppression by such large tumors.

An issue not directly addressed in this paper but raised by the significant evidence in the literature is whether anti-p185<sup>neu</sup> antibodies induce a functional block of p185<sup>neu</sup> receptor function,<sup>20</sup> down-regulate its expression on the cell surface,<sup>20,21</sup> impede the formation of p185<sup>neu</sup> homo- or heterodimers that spontaneously transduce proliferative signals to the cells,<sup>21,22</sup> and block its ability to bind ligands.<sup>23</sup> These antibodies also significantly suppress the growth of transplantable p185<sup>neu+</sup> tumors,<sup>24,25</sup> the natural onset of mammary carcinomas in Her-2/neu transgenic mice,<sup>21</sup> and delay tumor growth in patients with Her-2/neu positive tumors.<sup>26</sup> A reduced r-p185<sup>neu</sup> expression could be sufficient to reverse their transformed phenotype.<sup>20,21</sup>

The marked increase in serum IgG2a and IgA in ECD-IL1 $\beta$ p-immunized mice suggests a more finely divided reaction scenario wherein leukocytes infiltrating the tumor site may play an important effector role.<sup>27-29</sup> These antibody isotypes activate PMN and other cells to mediate antibody dependent cellular cytotoxicity (ADCC)<sup>25,30</sup> and complement-dependent cytotoxicity (IgG2a), and inhibit the growth of p185<sup>neu+</sup> tumor *in vivo*.<sup>25</sup> IgG and IgA may synergistically promote ADCC by PMN and other leukocytes<sup>25,31</sup> that massively infiltrate hyperplastic lesions. It is also possible that the concentration of secretory IgA may reach particularly high levels in the mammary gland, where their inhibitory activity is required.

## Material and methods

### Mice

Inbred BALB-neuT mice overexpressing the transforming rat Her-2/neu oncogene (neuT<sup>+</sup>/neuT<sup>-</sup>) driven by the mouse mammary tumor virus promoter were produced and screened for the presence of the transgene as previously described in detail.<sup>15</sup> Groups of individually tagged virgin BALB-neuT females bred under specific pathogen-free conditions by Charles River, Calco, Italy were treated in accordance with European Union and institutional guidelines. Since all 10 mammary glands of BALB-neuT females naturally undergo carcinogenic transformation with a definite progression,<sup>15</sup> these were inspected weekly, and tumor masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, when mice were killed for humane reasons. Except where otherwise specified, surviving BALB-neuT mice were killed at 33 weeks.<sup>15</sup> As some immunized mice do not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas per mouse was calculated as cumulative number of incident tumors per total number of BALB-neuT mice.

### DNA expression vectors and vaccination

The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA, USA) by deleting the

SV40 promoter, neomycin resistance gene and SV40 polyA. The sequence for the extracellular domain of transforming mutated rat p185<sup>neu</sup> was generated from the PCR product using the primers 3'-CGCAAGCTTCAT-CATGGAGCTGGC-5' and 3'-CGGAATTCGGGCTGGCTCTCTGCTC-5' and the primers 3'-CGCAAGCTTCATGGAGCTGGC-5' and 3'-ATGAATCTTTCCGCATCGTGTACTTCTTCCGG-5', respectively, as previously described.<sup>17</sup> PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hind*III and *Eco*RI and cloned into the multiple cloning site of the pCMV plasmid in order to obtain the ECD plasmid used in this work. The immunomodulatory IL1 $\beta$ p nonapeptide WQGEESNDK corresponding to amino acids 163-171 and the control non-active IL1 $\beta$ na nonapeptide EGTEKDQVS corresponding to amino acids 189-197 of the human IL-1 $\beta$  were cloned in-frame into *Eco*RI and *Xba*I sites obtained by incorporating two complementary and overlapping phosphorylated oligonucleotides encoding each of the two peptides with *Eco*RI and *Xba*I sites: 5'-AATTCGGTTCAGGGTGAAGAAAGTAACGATAAATAAT-3' (IL1 $\beta$ p-Forw) and 5'-CTAGATTATTTATCGTTACTTTC TTCACCCTGAACCG-3' (IL1 $\beta$ p-Rew); 5'-AATTCGGAAGGTACCGAAAAAGATCAGGTTAGTTAAT-3' (IL1 $\beta$ na-Forw) and 5'-CTAGATTAACCTACCTGATCTTTTTTCGGTACCTTCCG-3' (IL1 $\beta$ na-Rew). *Escherichia coli* strain DH5 was transformed with ECD, ECD-IL1 $\beta$ p and ECD-IL1 $\beta$ na plasmids and then grown in Luria-Bertani medium (Sigma, St Louis, MO, USA) as previously described.<sup>17</sup> Large-scale preparation of the plasmids was carried out by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA, USA). DNA was then precipitated, suspended in sterile saline at the concentration of 1 mg/ml and stored in aliquots at -20°C for subsequent use in immunization protocols. Plasmids were injected (100  $\mu$ g per injection) into the quadriceps muscle through a 28-gauge needle syringe. BALB-neuT mice were immunized at the 6th, 12th, 18th and 24th week.

### Morphologic analysis

Groups of three BALB-neuT mice were killed at the indicated times. For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4  $\mu$ m and stained with hematoxylin and eosin or Giemsa.

### Cell lines

TUBO and N202.1 cells are cloned cell lines established *in vitro* from lobular carcinomas that arose spontaneously in a BALB-neuT and in an FVB-neuN transgenic mouse, respectively.<sup>19</sup> N202.1A and N202.1E are two clones randomly derived from N202.1.<sup>32</sup> Cytofluorimetric analysis indicates that N202.1A expresses high levels of p185<sup>neu</sup>, whereas this expression is not detectable in N202.1E.<sup>32</sup> Both clones were cultured in DMEM (BioWhittaker Europe, Verviers, Belgium) supplemented with 20% FBS (Life Technologies, San Giuliano Milanese, Italy).

### Cytotoxicity assays

The cytotoxicity of lymphocytes from the mice in each group was tested immediately or after *in vitro* restimulation. Lymphocytes ( $1 \times 10^7$ ) were stimulated for 6 days with  $5 \times 10^5$  irradiated TUBO cells as previously described.<sup>33</sup> To get better stimulation, this basic design

was variously changed in the several repeats of the test. Other rat Her-2/neu expressing BALB/c target cells were also used as stimulator and target cells. Moreover, the suppressor activity of stimulator rat Her-2/neu BALB/c cells was ruled out by adding progressive numbers of third-party TUBO cells in mixed lymphocyte and allogeneic target cell interactions as previously described.<sup>34</sup> Cytotoxicity of fresh and restimulated lymphocytes was assayed in 4 and 18 h <sup>51</sup>Cr release assays, 48 and 72 h [<sup>3</sup>H]TdR release assays as previously described in detail.<sup>33,34</sup> In all these tests both TUBO cells and other rat Her-2/neu expressing BALB/c target cells were highly lysable by allogeneic cytotoxic T lymphocytes.

#### Cytofluorimetric evaluation of anti r-p185<sup>neu</sup> antibodies

Sera of six BALB-neuT mice immunized with ECD, ECD-IL1 $\beta$ p and ECD-IL1 $\beta$ na were collected at 33 weeks when they all display 10 palpable tumors and pooled. The sera of ECD-IL1 $\beta$ p immunized mice displaying only 1–4 palpable tumors at 33 weeks was separately collected. The control sera were a pool from six mice injected with saline only. The ability of sera to bind r-p185<sup>neu</sup> was evaluated by flow cytometry.  $2 \times 10^5$  N02.1A or N202.1E cells from *in vitro* cultures, washed twice with cold PBS supplemented with 2% BSA and 0.05% sodium azide, were stained in a standard indirect immunofluorescence procedure with 50  $\mu$ l of 1:10 dilution in PBS-azide-BSA of control or immune sera. A fluorescein-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) was used as second-step Ab. The cells were resuspended in PBS-azide-BSA containing 1 mg/ml of propidium iodide to gate out dead cells, and evaluated in a FACScan (Becton Dickinson). The specific N202.1A binding potential (Sbp) of the sera was calculated as follows: [(% positive cells with test serum) (fluorescence mean)] – [(% positive cells with control serum) (fluorescence mean)] X serum dilution, as previously described in detail.<sup>34</sup>  $5 \times 10^3$  viable cells were analyzed in each evaluation.

#### Serum concentration of Ig isotypes

The concentration of IgA, IgM, IgG1, IgG2a, IgG2b and IgG3a isotypes in pool of mice injected four times with saline only or immunized with ECD, ECD-IL1 $\beta$ p or ECD-IL1 $\beta$ na was determined by the radial immunodiffusion test (The Binding Site, Birmingham, UK).

#### Statistical analysis

Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test, those in tumor/mouse numbers by Wilcoxon's rank sum test and those in the number of tumor infiltrating cells by Student's *t* test.

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