

# A Fully Human Antitumor ImmunoRNase Selective for ErbB-2-Positive Carcinomas

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## ABSTRACT

We report the preparation and characterization of a novel, fully human antitumor immunoRNase (IR). The IR, a human RNase and fusion protein made up of a human single chain variable fragment (scFv), is directed to the ErbB-2 receptor and overexpressed in many carcinomas. The anti-ErbB-2 IR, named hERB-hRNase, retains the enzymatic activity of the wild-type enzyme (human pancreatic RNase) and specifically binds to ErbB-2-positive cells with the high affinity ( $K_d = 4.5$  nM) of the parental scFv. hERB-hRNase behaves as an immunoprototoxin and on internalization by target cells becomes selectively cytotoxic in a dose-dependent manner at nanomolar concentrations. Administered in five doses of 1.5 mg/kg to mice bearing an ErbB-2-positive tumor, hERB-hRNase induced a dramatic reduction in tumor volume. hERB-hRNase is the first fully human antitumor IR produced thus far, with a high potential as a poorly immunogenic human drug devoid of nonspecific toxicity, directed against ErbB-2-positive malignancies.

## INTRODUCTION

Immunotherapy is of great interest today as an effective strategy to manage cancer and as an alternative to chemotherapy. The Food and Drug Administration has approved several monoclonal antibodies as therapeutic agents to manage tumors, and an increasing number are undergoing clinical evaluation (1). Examples of approved antibodies are humanized or human-mouse chimeric monoclonal antibodies, such as anti-ErbB-2 trastuzumab (Herceptin) and anti-CD20 rituximab (Rituxan), widely used against breast cancer and non-Hodgkin's lymphoma, respectively (2). To enhance their clinical potential, antibodies also have been coupled to cytotoxic agents or radionuclides (3).

Immunotoxins (ITs), made up of antibodies or miniantibodies [single chain variable fragment (scFv)] fused to toxins, have been proposed as anticancer drugs during the past 2 decades (4, 5). These chimeric proteins combine the potent toxicity of toxins with the antigen specificity of antibodies. However, the murine nature of the immunomoiety, the plant or bacterial nature of the toxin moieties, and their high toxicity have greatly limited the therapeutic potential of ITs (6, 7), especially because of the occurrence of vascular leak syndrome (8–10). Although the development of humanized antibodies has alleviated some of these effects, the toxins themselves remain a problem.

An alternative, more recent strategy in anticancer immunotherapy is that based on immunoconjugates in which an RNase substitutes for the toxin (11). Mammalian RNases are not toxic to cells unless internalized; thus, these fusion proteins are not ITs but rather immunoprototoxins. We have proposed (12) to call them immunoRNases (IRs). IRs have been prepared with various RNases, each fused to a monoclonal antibody raised against cell receptors (12–15). However,

the IRs prepared thus far have a limited interest for immunotherapy, given the nonhuman origin of the antibody moiety used and the choice of the targeted receptors, such as the epidermal growth factor or the transferrin receptor (16, 17).

Because of its preferential expression in tumor cells (18) and its extracellular accessibility, an attractive target for immunotherapy is ErbB-2, a transmembrane tyrosine kinase receptor that is overexpressed on many carcinoma cells of different origin (19, 20), with a key role in the development of malignancy (21, 22). Furthermore, activated ErbB-2 is readily internalized, an event that can be mimicked by an antibody directed toward the receptor. Thus, an anti-ErbB-2 antibody can deliver an RNase into ErbB-2-overexpressing tumor cells. Such a strategy has been successfully tested using a murine scFv fused to a human RNase (12).

The use of antibody and RNase moieties of human origin for the preparation of fully human IRs is highly desirable to obtain effective and tumor-selective but also immunocompatible immunoagents.

Fully human scFvs recently have been generated with the phage display technology through the expression of large repertoires of antibody variable regions on filamentous phages after their fusion to a phage coat protein (23–25). Taking advantage of this powerful technique, we have isolated a novel human anti-ErbB-2 scFv (26) from a large phage display library (23) through a double-selection strategy performed on live cells. This scFv specifically binds to ErbB-2-positive cells, inhibits receptor autophosphorylation, is internalized in target cells, and strongly inhibits their proliferation (26).

We report here the construction and characterization of a fully human antitumor IR made up of the available human anti-ErbB-2 scFv (26) and a human RNase, which we have termed hERB-hRNase. This IR, to our knowledge the first human IR to be produced, may prove to be a valuable anticancer agent for therapy of ErbB-2-overexpressing carcinomas.

## MATERIALS AND METHODS

**Antibodies and Cell Cultures.** The antibodies used in the current study were the following: murine monoclonal antibody 9E10 directed against the myc tag protein (27), the IgG fraction from a rabbit anti-human pancreas RNase (HP-RNase) antiserum (Igtech, Salerno, Italy) purified by affinity chromatography of the antiserum on protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ), horseradish peroxidase-conjugated anti-His antibody (Qiagen, Valencia, CA), horseradish peroxidase-conjugated goat antirabbit immunoglobulin antibody (Pierce, Rockford, IL), horseradish peroxidase-conjugated rabbit antimouse immunoglobulin antibody (Pierce), and FITC-conjugated sheep antirabbit immunoglobulin antibody (Dako, Glostrup, Denmark).

The MDA-MB361 (provided by Dr. N. Normanno, Cancer Institute of Naples, Italy) and the SKBR3 cell lines from human breast tumor and the A431 cell line from human epidermoid carcinoma (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Life Technologies, Rockville, MD). The MDA-MB453 cell line from human breast tumor (a gift of H. C. Hurst, ICRF, London, UK) and the TUBO cell line from a BALB-neu T mouse-derived mammary lobular carcinoma (provided by Dr. G. Forni, University of Turin, Italy) were grown in DMEM (Life Technologies). Media were supplemented with 10% fetal bovine serum (20% for TUBO cells), 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin (all from Life Technologies).

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**Construction of the Chimeric cDNA Encoding hERB-hRNase.** A cDNA coding for HP-RNase (28) was engineered by two successive PCRs. Upstream primers (termed A and B) were used to incorporate the *NotI* restriction site at the 5' end and a spacer sequence, whereas a downstream primer (C) was used to introduce a *NotI* site at the 3' end. In the first reaction, primers B and C were used. The oligonucleotide B sequence (5'-GGCCCGAAGGCGGCAGCAAAGAATCTAGAGCTAAAAA-3') encodes the COOH-terminal half of the spacer; the oligonucleotide C sequence (5'-ATAAGAATGCGGCCGCA-GAGTCTTCAACAGACG-3') contains the *NotI* restriction site. In the second reaction, primers A and C were used. Oligonucleotide A (5'-ATAAGAATGCGGCCGCAAGCGGCCGCGAAGGCGG-3') encodes the N-terminal half of the spacer preceded by the *NotI* site sequence. The PCR fragment then was digested with *NotI* (New England Biolabs, Beverly, MA) and cloned into the corresponding site of pHEN2 vector (29) downstream to the sequence encoding the available human anti-ErbB-2 scFv (26). The correct directional insertion of the RNase gene in the *NotI* site was assessed by PCR using the forward primer A in combination with a reverse oligonucleotide corresponding to a vector sequence positioned downstream to the *NotI* site (5'-TGAATTT-TCTGTATGAGG-3').

Sequence analyses confirmed the expected DNA sequence. The assembled gene then was cloned in a T7 promoter-based *Escherichia coli* expression vector (pET22b+).

**Expression and Purification of hERB-hRNase.** Cultures of *E. coli* BL21 (DE3), previously transformed with the recombinant pET22b(+) expression vector, were grown at 37°C in LB medium containing 50 µg/ml ampicillin until the exponential phase was reached. The expression of soluble IR (henceforth called hERB-hRNase) was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM in the cell culture, which then was grown at room temperature overnight. Cells were harvested by centrifugation at 6000 rpm for 15 min, and the periplasmic extract was obtained as described previously (26) and incubated with the 4-mercapto-ethyl-pyridine HyperCel matrix (BioSeptra, Cergy-Saint-Christophe, France) for 2 h at room temperature. After extensive washes with 50 mM Tris-HCl (pH 8.0), two additional wash steps were performed: the first with H<sub>2</sub>O and the second with 25 mM sodium caprylate in 50 mM Tris-HCl (pH 8.0). The 4-mercapto-ethyl-pyridine HyperCel resin then was equilibrated in 50 mM Tris-HCl (pH 8.0) before elution of the protein with 50 mM sodium acetate (pH 4.0). The sample was immediately adjusted to pH 7.0, diluted with B-PER buffer (Pierce), and loaded on an immobilized-metal affinity chromatography column using a cobalt-chelating resin (TALON; Clontech, Palo Alto, CA) to achieve further purification. Wash and elution steps were performed according to the manufacturer's recommendations. The purity of the final preparation was evaluated by SDS-PAGE, followed by Coomassie staining and Western blot analysis with anti-HP-RNase immunoglobulins, followed by goat antirabbit horseradish peroxidase-conjugated IgG.

**RNase Activity Assays.** RNase activity was tested as described previously (30) on yeast RNA (8 mg/ml). RNase zymograms, carried out on SDS-PAGE electropherograms, were performed as described previously (31).

**Binding Assays.** ErbB-2-positive SKBR3, MDA-MB361, MDA-MB453, and TUBO cells and ErbB-2-negative A431 control cells, harvested in non-enzymatic dissociation solution (Sigma, St. Louis, MO), were washed and transferred to U-bottomed microtiter plates (1 × 10<sup>5</sup> cells/well). After blocking with PBS containing 6% BSA, the plates were incubated with purified immunoagents in ELISA buffer (PBS/BSA 3%) for 90 min. After centrifugation and removal of supernatants, pelleted cells were washed twice in 200 µl of ELISA buffer, resuspended in 100 µl of the same buffer, and incubated for 1 h with either rabbit anti-HP-RNase IgG (for the detection of hERB-hRNase) or with murine anti-myc monoclonal antibody (for scFv detection), followed by peroxidase-conjugated antirabbit or antimouse IgG (Pierce), respectively, according to the source of the primary antibody. After 1 h, the plates were centrifuged, washed with ELISA buffer, and reacted with 3,3',5,5'-tetramethylbenzidine (Sigma). Binding values were determined from the absorbance at 450 nm and reported as the mean of at least three determinations (SD ≤ 5%).

**Internalization of hERB-hRNase.** Cells grown on coverslips to 60% confluency were incubated with the immunoagent (20 µg/ml) for 16 h at 37°C. Cells then were washed, fixed, and permeabilized as described elsewhere (26). Intracellular IR was detected with a rabbit anti-HP-RNase antibody, followed

by FITC-conjugated sheep antirabbit antibody. Optical confocal sections were taken using a confocal microscope (LSM 510; Zeiss, Oberkochen, Germany).

**Cytotoxicity Assays.** Cells were seeded in 96-well plates (150 µl/well); SKBR3, MDA-MB361, TUBO, and MDA-MB453 cells were seeded at a density of 1.5 × 10<sup>4</sup>/well, and A431 cells were seeded at a density of 5 × 10<sup>3</sup>/well. Cell survival was expressed as percentage of viable cells in the presence of the protein under test, with respect to control cultures grown in the absence of the protein.

**Stability of the IR.** The stability of hERB-hRNase was determined by incubating the protein at a concentration of 0.04 mg/ml in either human or murine serum at 37°C for 24 or 48 h. At the end of incubation the samples were tested using the analytical tests described previously.

**In Vivo Antitumor Activity.** All of the experiments were performed with 6-week-old female BALB/cAnNCrIBR mice (Charles River Laboratories, Wilmington, MA). TUBO cells (5 × 10<sup>5</sup>) were suspended in 0.2 ml sterile PBS and injected s.c. (day 0) in the right paw. At day 10, tumors were clearly detectable (at least 15 mm<sup>3</sup> in volume). At day 11, hERB-hRNase, dissolved in PBS, was administered five times at 72-h intervals peritumorally or i.p. to two groups of five mice at doses of 1.5 mg/kg of body weight. Equimolar doses of native HP-RNase or anti-ErbB-2 scFv were administered peritumorally to other two groups as controls. Another group of control animals was treated with identical volumes of sterile PBS. At day 45, blood samples were taken and tested to obtain the main hematologic parameters. During the treatment period, tumor volumes (V) were measured and calculated using the formula of rotational ellipsoid  $V = A \times B^2/2$  (A is axial diameter and B is rotational diameter). All of the mice were maintained at the animal facility of the Department of Cellular and Molecular Biology and Pathology, University of Naples Federico II. The experiments on animals described here were conducted in accordance with accepted standards of animal care and the Italian regulations for the welfare of animals used in studies of experimental neoplasia. The School of Medicine Institutional Committee on animal care approved the study.

## RESULTS

**Construction and Purification of hERB-hRNase.** The anti-ErbB-2 fully human IR was prepared as follows: the cDNA encoding HP-RNase was amplified by two successive PCRs (see "Materials and Methods"), which added a spacer sequence encoding a peptide of 11 residues designed to separate the RNase (28) and scFv moieties in the fusion protein. The resulting product was cloned in the expression vector pHEN2 (29), downstream to the sequence encoding the available human anti-ErbB-2 scFv (26), and in *NcoI/NotI* sites. The resulting construct encoding the IR, named hERB-hRNase, is shown in Fig. 1. It includes a 15-residue linker made up of glycine and serine residues (SSGGGGSGGGSGGS) interposed between V<sub>H</sub> and V<sub>L</sub> chains, an 11-residue spacer (AAASGGPEGG) inserted between the antibody fragment and the RNase, and a COOH-terminal hexahistidine tag. The chimeric cDNA was fully sequenced and re-cloned in a T7 promoter-based *E. coli* expression vector (pET22b+) equipped with a pel B signal sequence for expression of hERB-hRNase as a soluble protein in the periplasmic space.

**Characterization of hERB-hRNase.** The purified IR was analyzed by Western blot analysis with an anti-His and an anti-HP-RNase

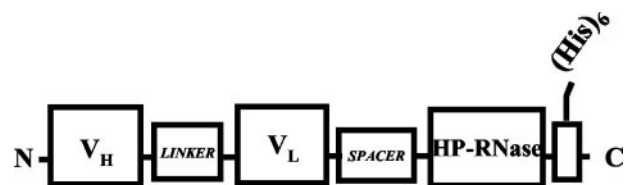


Fig. 1. Schematic representation of the human immunorNase hERB-hRNase. V<sub>H</sub> and V<sub>L</sub>, the variable domains of heavy and light chains, respectively, of the anti-ErbB-2 single chain variable fragment (scFv); LINKER, the 15-residue junction peptide SS(G<sub>4</sub>S)<sub>2</sub>GGS; SPACER, the peptide AAASGGPEGG connecting the scFv and the RNase moieties; HP-RNase, human pancreatic RNase; and (His)<sub>6</sub>, a 6-residue His tag.

antibody. By both analyses, an immunoreactive band of the expected size, approximately  $M_r$  46,000, was visualized (Fig. 2).

The fusion protein then was tested for enzymatic activity by a zymogram developed using yeast RNA as a substrate. As illustrated in Fig. 2, a single active band was detectable, corresponding to the size of hERB-hRNase. The ribonucleolytic activity of the purified IR was further tested with the acid-insoluble RNA precipitation assay (30), by which the chimeric protein was found to have a specific activity of  $950 \pm 25$  units/nmol. This result was confirmed for several preparations of the recombinant fusion protein. Because the specific activity of wild-type HP-RNase is  $\sim 1100 \pm 20$  units/nmol, we can conclude that the chimeric protein retains  $\sim 90\%$  of the activity of the parental RNase molecule.

The ability of hERB-hRNase to specifically recognize ErbB-2 was evaluated by ELISA, performed as described in "Materials and Methods," using SKBR3 cells from human breast cancer, which express high levels of ErbB-2. As a control we used A431 cells from human epidermoid carcinoma, which express the receptor at low levels. The results, shown in Fig. 3, indicate that the IR binds with high affinity to SKBR3 cells, whereas no significant binding to A431 cells was detected. The apparent binding affinity of hERB-hRNase for the ErbB-2 receptor (*i.e.*, the concentration corresponding to half-maximal saturation) was found to be 4.5 nM, almost identical to that obtained with the parental scFv (4 nM; see Fig. 3).

These results demonstrate that the antibody and RNase moieties maintain their biological functions in the chimeric protein.

**Internalization of hERB-hRNase by SKBR3 Cells.** It has been reported that the human anti-ErbB-2 scFv, previously isolated in our laboratory, undergoes receptor-mediated endocytosis in SKBR3 cells

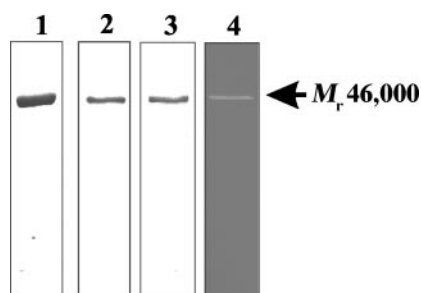


Fig. 2. SDS-PAGE analyses of purified hERB-hRNase. Lane 1, hERB-hRNase eluted from the cobalt-chelating affinity chromatography (Coomassie Blue staining); Lanes 2 and 3, Western blot analyses of the sample as in Lane 1 using anti-human pancreatic-RNase IgGs (Lane 2) or an anti-His antibody (Lane 3); Lane 4, zymogram of the sample as in Lane 1 using yeast RNA as a substrate.

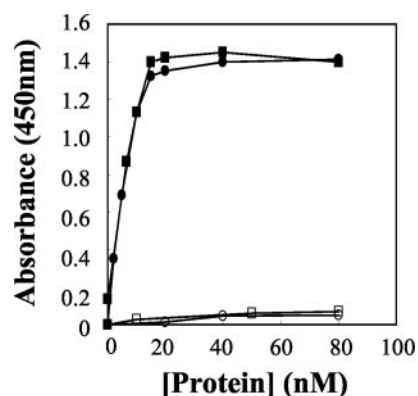


Fig. 3. Binding tests of hERB-hRNase to ErbB-2-positive (SKBR3) and ErbB-2-negative (A431) cell lines. SKBR3 cells (black symbols) and A431 cells (white symbols) were tested by ELISA with hERB-hRNase (squares) or with the anti-ErbB-2 single chain variable fragment (circles) as a control.

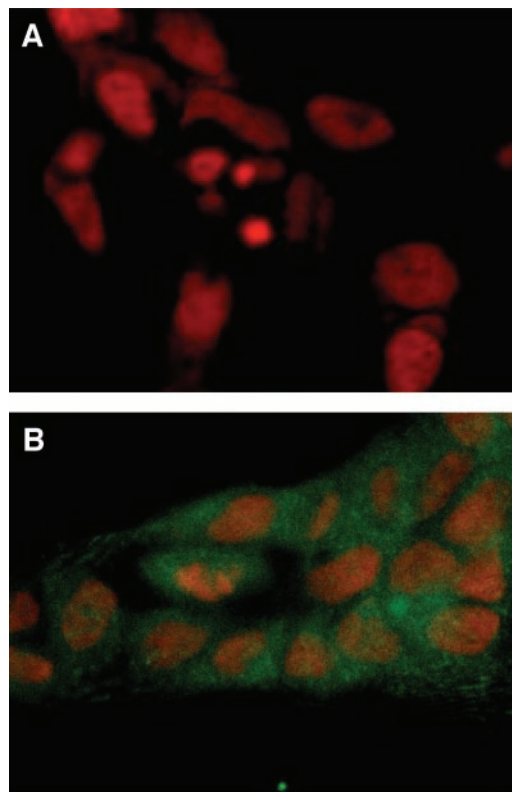


Fig. 4. Internalization of hERB-hRNase in SKBR3 cells as visualized by confocal microscopy. Cells were incubated in the absence (A) or in the presence of the immunorNase for 16 h (B); magnification 1:200.

(26). To test whether the scFv in the fusion protein could provide a useful vehicle to deliver the RNase into the cytosol, tumor target cells were incubated with hERB-hRNase for 16 h at 37°C. After extensive washes, cells were fixed and permeabilized as described previously (26). Internalized IR was visualized with anti-HP-RNase antibody, followed by sheep antirabbit FITC-conjugated antibody. As shown in Fig. 4, a strong intracellular staining was visualized (Fig. 4B) by confocal microscopy.

**Cytotoxic Effects of hERB-hRNase on Tumor Cells.** The purified hERB-hRNase then was tested for its effects on ErbB-2-positive and ErbB-2-negative cell survival. For the latter cell type, A431 cells from human epidermoid carcinoma were used; as antigen-positive cells, we used SKBR3, MDA-MB361, and MDA-MB453 derived from human breast carcinomas and TUBO cells from a BALB-neu T mouse-derived mammary lobular carcinoma (32). Cells were plated in the absence or in the presence of increasing concentrations of hERB-hRNase, and after a 72-h incubation, cell survival was measured by counting trypan blue-excluding cells.

As shown in Fig. 5, hERB-hRNase was found to be selectively cytotoxic in a dose-dependent manner on all of the antigen-positive cell lines tested. The values of  $IC_{50}$  (*i.e.*, the concentration capable of reducing cell viability by 50%) were found to be 12.5, 47, 52, and 60 nM for SKBR3, MDA-MB361, MDA-MB453, and TUBO cells, respectively. When the IR was tested on A431 control cells, no effects on their proliferation were observed (see Fig. 5). Moreover, no effects on cell survival were detected when native HP-RNase was tested on ErbB-2-positive cells at concentrations up to 1  $\mu$ M (*i.e.*, at doses of RNase up to 20-fold higher than the highest dose of RNase present in the IR samples tested on the cell cultures). These results indicate that (a) the RNase moiety in the IR has no effect on cell proliferation unless driven inside the cells by the immune moiety; and (b) hERB-

hRNase is able to finely discriminate between target and nontarget cells and to specifically induce the death of target cells.

The stronger cytotoxicity of hERB-hRNase on SKBR3 and MDA-MB361 cells compared with that observed on MDA-MB453 cells could be related to their different level of ErbB-2, reported to be sixfold lower in the latter cells (12, 33). By ELISA we confirmed a lower level of ErbB-2 immunoreactivity in MDA-MB453 cells compared with SKBR3 and MDA-MB361 cells and found a similar low ErbB-2 content on TUBO cells (data not shown). These results indicate that there is a positive correlation between the expression levels of ErbB-2 on a particular cell type, the extent of binding of hERB-hRNase to those cells, and their sensitivity to its cytotoxic action.

**Stability of hERB-hRNase.** The stability of immunoagents at 37°C is a critical factor for their potential as therapeutic agents. The stability of hERB-hRNase in human or murine serum at 37°C for up to 48 h was analyzed by monitoring its integrity as a protein and as functional bioeffector. Following an incubation in serum for 24 or 48 h at 37°C, the percentage of undegraded and enzymatically active IR was determined by Western blot analysis with anti-His tag antibody and zymogram analyses, respectively. By using a Phosphorimager, the intensity of the electrophoretic bands obtained with treated hERB-hRNase was expressed as the percentage of the signal given by the untreated protein. We found that after 24 or 48 h, the percentage of undegraded and enzymatically active hERB-hRNase was  $100 \pm 5\%$ .

The binding properties of the IR after incubation in serum were assessed by ELISA on ErbB-2-positive cells and expressed as the concentration corresponding to half-maximal saturation. It was found that the protein incubated in human or murine serum conserved a value of half-maximal saturation of 5 nM, virtually identical to that measured for the protein before incubation (see above).

Finally, hERB-hRNase was found to fully retain its cytotoxic activity after incubation at 37°C for up to 48 h. The  $IC_{50}$  values of the incubated samples tested on SKBR3 cells (see above) were within  $\pm 10\%$  of the values measured before incubation.

These results clearly indicate that the IR is stable under the examined physiologic-like conditions.

**In Vivo Antitumor Activity of hERB-hRNase.** For *in vivo* studies, the ErbB-2-positive tumor cell line TUBO of murine origin (see above) was used. As shown in Fig. 6, the hERB-hRNase treatment of mice bearing TUBO tumors with five doses of 1.5 mg/kg of hERB-hRNase injected peritumorally induced a dramatic reduction (86%) in tumor volume. Similar results were obtained when the protein was administered systemically by i.p. injections. This suggests that the protein is stable in the bloodstream and is able to permeate tumor masses. Contrarily, the anti-ErbB-2 scFv and native HP-RNase injected peritumorally showed no significant effects on tumor growth (see Fig. 6). During the treatment period, the animals did not show

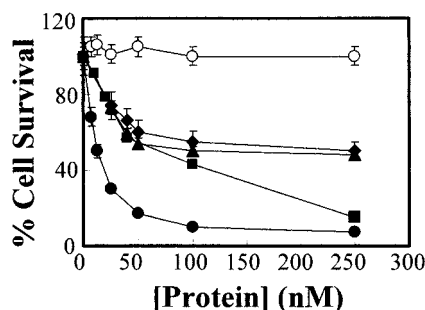


Fig. 5. Effects of hERB-hRNase on cell survival. Dose-response curves of SKBR3 (●), MDA-MB361 (■), MDA-MB453 (▲), TUBO (◆), and A431 cell lines (○) on treatment for 72 h with hERB-hRNase.

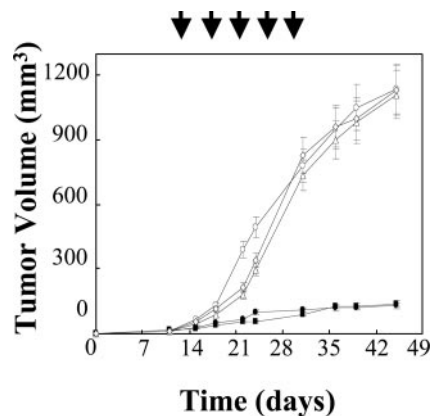


Fig. 6. *In vivo* suppression of tumor growth by hERB-hRNase. Tumor growth was followed in mice inoculated s.c with  $5 \times 10^5$  TUBO mammary carcinoma cells. Treated animals were injected i.p. (●) or in the peritumoral area (■) with hERB-hRNase at doses of 1.5 mg/kg body weight. Control animals were treated with sterile PBS solution (◇), equimolar doses of the anti-ErbB-2 single chain variable fragment (△), or human pancreatic-RNase (○) administered peritumorally. Injections were repeated five times at 72-h intervals as indicated by the arrows.

signs of wasting or other visible signs of toxicity. Their main hematologic parameters were those of normal mice (data not shown).

## DISCUSSION

Immunotherapy has been demonstrated to be a valuable approach in anticancer therapy. As previously indicated, ITs directed to cell surface molecular targets have been shown to have a therapeutic potential. However, they also have limitations, mainly represented by nonspecific toxicity related to vascular leak syndrome and by the immunogenicity of their bacterial or plant toxins (8–10).

To circumvent these problems, IRs (*i.e.*, chimeric proteins made up of RNases fused to an antireceptor antibody or scFv) have been prepared (13–15). These IRs were cytotoxic to tumor cells at nanomolar concentrations. However, the choice of epidermal growth factor or transferrin receptors as targets for the antibody, as well as the murine origin of the antibodies used, sets a limit in the use of these reagents as therapeutic drugs.

ErbB-2 is an attractive tumor target because of its specific localization on many tumor cells of different origin, its extracellular accessibility, and its high expression levels in many carcinomas (19, 20). Furthermore, its high expression in carcinomas has been reported to be a clear sign of negative prognosis (21, 22).

Here, we report the construction, characterization, and antitumor activity of the first fully human IR targeting the ErbB-2 receptor. In this novel fusion protein, named hERB-hRNase, the human scFv and human RNase moieties preserve their biological actions. In contrast with previous reports (11), the IR activity as an RNase is nearly that of the native, free enzyme. Likewise, the IR binds to target cells as selectively and effectively as the parental, free scFv.

As for the RNase fused in the IR construct, it is mandatory, for assigning to the IR the role and the action of an immunoprototoxin, that the RNase *per se* is not a cytotoxic agent. Our work has shown that the HP-RNase used in hERB-hRNase is not *per se* a cytotoxic agent, neither *in vivo* nor *in vitro*, but becomes cytotoxic on internalization. This may not be surprising because it has been reported that when nontoxic RNases, such as RNase A, are artificially introduced into frog oocytes, they become powerful cytotoxic agents (34).

It is the ability of the parental scFv to be internalized by ErbB-2-overexpressing cells fully preserved in the fusion protein, which allows the RNase to enter the cytosol and kill target cells.

It should be noted that the scFv alone was found to inhibit the

proliferation of SKBR3 cells with an  $IC_{50}$  value of 200 nM (26). This indicates that the IR, for which an  $IC_{50}$  value of 12.5 nM was obtained, is a more effective anticancer agent. This result can only be attributed to the additional toxic action of the internalized RNase because the RNase alone has no effect on cell viability.

It may be of interest that hHERB-hRNase has been found to be stable for at least 48 h when incubated at 37°C in human or murine serum. This feature of the IR adds to its potential as a promising antitumor drug. Even more interesting are the results of *in vivo* assays of hHERB-hRNase antitumor action carried out on mice inoculated with TUBO ErbB-2-positive tumor cells, which produce tumors similar to the alveolar-type human lobular mammary carcinomas (35). These experiments proved that low doses of hHERB-hRNase also are effective *in vivo*. Treatment of mice inoculated with TUBO cells with only five doses of IR (1.5 mg/kg body weight) administered either peritumorally or systemically strongly inhibited the growth of TUBO tumors in mice. Such effect again can be attributed to the fusion protein as a whole because the two moieties, the anti-ErbB-2 scFv and HP-RNase, when administered separately to mice bearing the same tumor, were not found to be effective in inhibiting tumor growth.

To our knowledge, hHERB-hRNase is to date the first fully human IR to be constructed and tested with satisfactory results *in vitro* and *in vivo*. Its fully human nature, combined with its stability and its selective cytotoxic action on target cells, could make it a precious tool in the therapy of human mammary carcinomas.

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