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A NOVEL FULLY HUMAN ANTITUMOR IMMUNORNASE TARGETING ErbB2-POSITIVE TUMORS

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RINGRAZIAMENTI E DEDICHE

SUMMARY

A second generation anti-ErbB2 ImmunoRNase, called Erb-hcAb-RNase, was obtained by the fusion of Erb-hcAb, a human compact anti-ErbB2 antibody, with human pancreatic ribonuclease (HP-RNase or RNase 1).

We show herein that Erb-hcAb-RNase retains the enzymatic activity of human pancreatic RNase and specifically binds to ErbB2-positive cells with an affinity comparable to that of the parental Erb-hcAb. Moreover, this novel immunoRNase is endowed with an effective and selective antiproliferative action for ErbB2-positive tumor cells both in vitro and in vivo. Its antitumor activity is more potent than that of the parental Erb-hcAb as the novel immunoconjugate has acquired RNase-based cytotoxicity in addition to the inhibitory growth effects, antibody-dependent and complement-dependent cytotoxicity of the compact antibody.

Erb-hcAb-RNase could be a promising candidate for the immunotherapy of ErbB2-positive tumours as it combines the advantages of the first generation scFv-based immunoRNase with those of a fully functional antibody.

RIASSUNTO

Una ImmunoRNasi anti-ErbB2 di seconda generazione, chiamata Erb-hcAb-RNasi, è stata ottenuta mediante fusione dell'anticorpo compatto anti-ErbB2, Erb-hcAb, con la ribonucleasi pancreatica umana (HP-RNasi o RNasi 1).
In questo lavoro dimostriamo che Erb-hcAb-RNasi conserva l'attività enzimatica della ribonucleasi pancreatica umana e lega specificamente cellule ErbB2 positive con una affinità paragonabile a quella dell'anticorpo di origine Erb-hcAb. Inoltre, la nuova immunoRNasi possiede un'attività citotossica per cellule tumorali ErbB2-positive sia in vitro che in vivo. La sua attività antitumorale è più potente di quella dell'anticorpo di origine Erb-hcAb dato che il nuovo immunoconiugato ha acquisito la citotossicità mediata dalla ribonucleasi oltre agli effetti di inibizione della crescita ed alla citotossicità dipendente da anticorpo e da complemento dell'anticorpo di origine Erb-hcAb. Il costrutto Erb-hcAb-RNasi potrebbe essere un promettente candidato da impiegare nell'immunoterapia dei tumori ErbB2-positivi poichè combina i vantaggi della prima generazione di immunoRNasi prodotta con scFv, con quelli di un anticorpo dotato di funzioni effettrici.

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INTRODUCTION

Immunotherapy is a precious strategy to overcome the limits of the conventional anti-cancer treatments. Indeed, targeting cancer cells via antibodies specific for tumor associated surface proteins could fulfil the lack of selectivity of radiotherapy and chemotherapy, and is a new interesting biomedical approach as it combines the rational drug design with the progress in understanding cancer biology.

To date, several humanized monoclonal antibodies have achieved FDA approval, and an increasing number are undergoing clinical evaluation (1-4) (Table 1). A successful example of an approved humanized antibody is represented by Herceptin, the only humanized antibody widely used against ErbB2 positive carcinomas for immunotherapy (5).

ErbB2 (Figure 1) is an attractive target for immunotherapy, as it is a transmembrane tyrosine kinase receptor, overexpressed on tumor cells of different origin, with a key role in the development of malignancy (6). Herceptin is currently used with success for breast cancer therapy; however, it can engender cardiotoxicity and a high fraction of breast cancer patients are resistant to Herceptin-treatment (7,8).

Furthermore, also carcinomas with a high expression of ErbB2, such as non-small cell lung carcinoma, gastric and prostatic tumours, have been found to be resistant or much less sensitive to Herceptin treatment (9,10).

A significant addition to the anticancer arsenal has been the construction of a new anti-ErbB2 immunoagent derived from a human, per se cytotoxic scFv, named Erbicin (11), and a human Fc domain from a human IgG1. This led to a fully human antitumour antibody, designed to be a reduced version of an IgG, with the antiproliferative effect of the scFv moiety on tumour target cells, combined with the ability of the Fc moiety to induce both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

The engineered antibody has been called Erb-hcAb (Erbicin-human-compact Antibody) (figure 2) for its "compact" size (100 kDa), compared with the full size (155 kDa) of a natural IgG. The smaller size should promote an increased extravascular diffusion and tumor penetration.

It has been reported (12) that Erb-hcAb is capable of selective binding to malignant ErbB2-positive cells and of inhibiting their growth in vitro and in vivo, with no effects on ErbB2-negative cells. Moreover, Erb-hcAb is endowed with both ADCC and CDC cytotoxic effects.

Table 1. Monoclonal antibodies approved for therapeutic use.

Generic name	Trade name	Antibody format	Antigen	Approved indication	FDA approval	EMEA approval	Sponsor
Muromomab	Orthoclone	murine IgG2a	CD3	Allograft rejection in allogeneic renal transplantation	6/19/86	NA	Ortho Biotech Inc
Abciximab	Reopro	chimeric Fab	GPIIb/IIIa receptor	Prevention of cardiac ischemic complications	12/16/93	NA	Centocor
Rituximab	Rituxan	chimeric IgG1κ	CD20	Non-Hodgkin's Lymphoma, chronic lymphocytic leukemia and rheumatoid arthritis	11/26/97	6/2/98	Genentech and Biogen Idec
Daclizumab	Zenapax	humanized IgG1κ	IL-2Rα	Prophylaxis of acute organ rejection in renal transplants	12/10/97	2/26/99	Hoffman-La Roche
Basiliximab	Simulect	chimeric IgG1κ	IL-2Rα	Prophylaxis of acute organ rejection in renal transplantation	5/12/98	10/9/98	Novartis
Palivizumab	Synagis	humanized IgG1κ	RSV F protein	Respiratory syncytial virus infection	6/19/98	8/13/99	Medimmune
Infliximab	Remicade	chimeric IgG1κ	TNFα	Crohn's disease and rheumatoid arthritis *etc*	8/24/98	8/13/99	Centocor
Trastuzumab	Herceptin	humanized IgG1κ	Her2	Breast cancer	9/25/98	8/28/00	Genentech
Gemtuzumab Ozogamicin	Mylotarg[1]	calicheamicin-humanized IgG4κ	CD33	Acute myeloid leukemia	5/17/00	NA	Wyeth/Pfizer
Alemtuzumab	Campath	humanized IgG1κ	CD52	B-cell chronic lymphocytic leukemia	5/7/01	7/6/01	Ilex/Genzyme
Ibritumomab Tiuxetan	Zevalin	Y^{90}-murine IgG1κ	CD20	B-cell non-Hodgkin's lymphoma	2/19/02	1/16/04	Biogen Idec/Spectrum
Adalimumab	Humira	human IgG1κ	TNFα	Rheumatoid arthritis and Crohn's disease *etc*	12/31/02	9/1/03	Abbott
Omalizumab	Xolair	humanized IgG1κ	IgE	Moderate to severe persistent asthma	6/20/03	10/25/05	Genentech
Tositumomab	Bexxar	I^{131}-murine IgG2aλ	CD20	Non-Hodgkin's lymphoma	6/27/03	NA	Corixa/GSK
Efalizumab	Raptiva[2]	Humanized IgG1κ	CD11a	Moderate to severe plaque psoriasis	10/27/03	9/20/04	Genentech
Cetuximab	Erbitux	chimeric IgG1κ	EGFR	Head and Neck cancer, colorectal cancer	2/12/04	6/29/04	ImClone/BMS/ Merck kGa
Bevacizumab	Avastin	humanized IgG1κ	VEGF-A	Various solid tumors	2/26/04	1/12/05	Genentech
Natalizumab	Tysabri	humanized IgG4κ	α4-integrin	Multiple sclerosis and Crohn's disease	11/23/04	6/27/06	Biogen Idec/Elan
Ranibizumab	Lucentis	humanized Fab	VEGF-A	Age-related macular degeneration	6/30/06	1/22/07	Genentech
Panitumumab	Vectibix	human IgG2κ	EGFR	Metastatic colorectal carcinoma	9/27/06	12/19/07	Amgen
Eculizumab	Soliris	humanized IgG2/4κ	C5	Paroxysmal nocturnal hemoglobinuria	3/16/07	6/20/07	Alexion
Certolizumab Pegol	Cimzia	Peglated humanized Fab	TNFα	Crohn's disease and rheumatoid arthritis	4/22/08	10/1/09	UCB, Inc
Golimumab	Simponi	human IgG1κ	TNFα	Rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis	4/24/09	10/1/09	Centocor Ortho Biotech
Canakinumab	Ilaris	human IgG1κ	IL-1β	Cryopyrin-associated periodic syndromes	6/17/09	10/23/09	Novartis
Ustekinumab	Stelara	human IgG1κ	IL-12/ IL-23	Plaque psoriasis	9/25/09	1/16/09	Centocor Ortho Biotech
Ofatumumab	Arzerra	human IgG1κ	CD20	Chronic lymphocytic leukemia	10/26/09	NA	Glaxo Grp Ltd
Tocilizumab	Actemra	humanized IgG1κ	IL-6R	Rheumatoid arthritis	1/8/10	NA	Roche/Chugai
Denosumab	Prolia	human IgG2κ	RANK ligand	Postmenopausal women with risk of osteoporosis	06/02/10	05/28/10	Amgen
Catumaxomab	Removab	murine/rat hybrid IgG	EpCAM and CD3	Intraperitoneal treatment of malignant ascites in patients with EpCAM-positive carcinomas		04/20/09	TRION Pharma
Edrecolomab	Panorex[3]	murine IgG2a	EpCAM	Colon cancer			Wellcome/ Centocor
I^{131}-TNT	Cotara[4]	I^{131}-chimeric IgG1	DNA	Lung cancer			MediPharm Biotech
Nimotuzumab	Theracim[5]	humanized IgG1	EGFR	Nasopharyngeal carcinomas and head and neck tumors			CIM/CIMAB/ YM Bioscience

[1] On June 21, 2010, Pfizer announced the voluntary withdrawal from the US market of Mylotarg® (gemtuzumab ozogamicin) for patients with acute myeloid leukemia, at the request of the US FDA after results from a recent clinical trial raised new concerns about the product's safety and the drug failed to demonstrate clinical benefit to patients enrolled in trials.
[2] Efalizumab (Raptiva®) has been withdrawn from US market due to side effect. As of 06/09/09, it is no longer available in the United States. It also has been withdrawn from EU market since 08/05/09.
[3] Edrecolomab (Panorex®) was approved in Germany in 1995, but was subsequently withdrawn from market.
[4] I^{131}-TNT (Cotara®) was approved in China in 2003.
[5] Nimotuzumab (Theracim®) was approved in Cuba, Argentina, Colombia, India and China in 2005 and 2006.

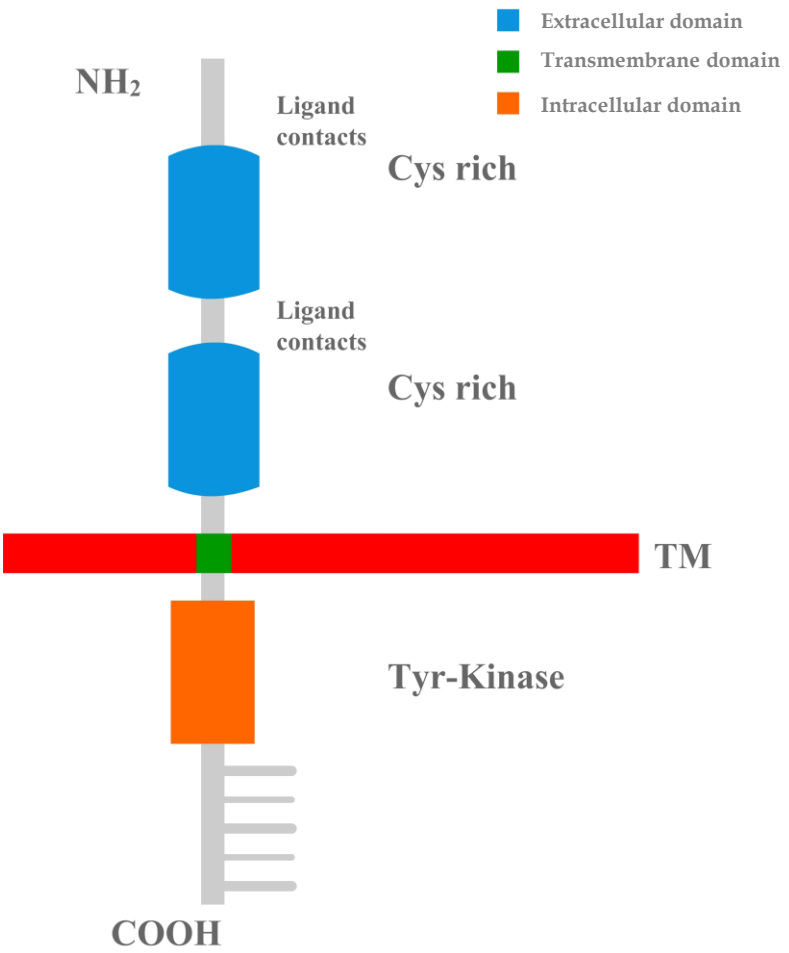

Figure 1. Schematic representation of ErbB2 receptor

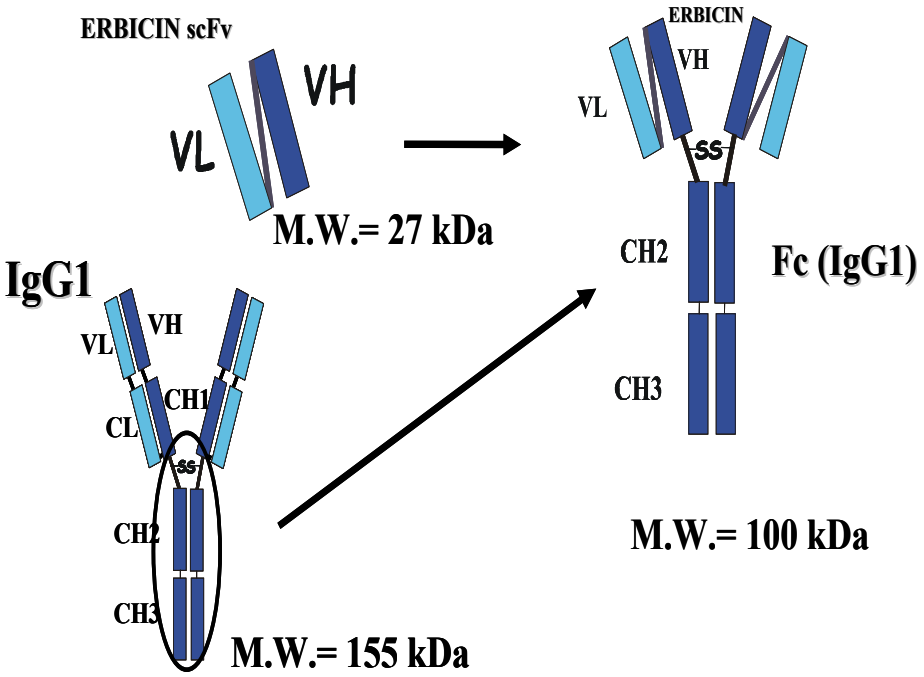

Figure. 2-The construction of Erb-hcAb, a human compact anti-ErbB2 antibody. *Left*: Erbicin, the parental anti-ErbB2 scFv. *Right*: Erb-hcAb. VH and VL are the heavy and light chain variable domains, respectively, as derived from Erbicin. H, the hinge region with disulfide bridges. CH2 and CH3 are the heavy chain constant domains of a human IgG1. *Bottom*: a full size IgG1

More recently, it has been shown that Erb-hcAb does not display the cardiotoxic effects of Herceptin in vitro on rat cardiomyocytes and in vivo on a mouse model (13), whereas Herceptin was found to be strongly toxic.

This difference was found to be due to the different mechanism of action of the two antibodies: Herceptin, at difference with Erb-hcAb, induces apoptosis in cardiac cells (13). Finally, Erb-hcAb is active in vitro and in vivo against some Herceptin-resistant, ErbB2 positive breast cancer cell lines (14).

Targeted therapy can be accomplished also by using monoclonal antibodies (MAbs) equipped with radionuclides or toxins (15,16). Immunotoxins are anticancer agents made up of a recombinant antibody or an antibody fragment directed towards a unique cell surface protein and a potent bacterial toxin capable of inducing the death of target cells (17,18).

However, problems have been encountered in clinical trials, especially for the toxicity and immunogenicity of the bacterial or plant toxins used for the immunotoxins (19,20).

As an alternative to immunotoxins, ImmunoRNases (IR) have been proposed as more immuncompatible immunoagents. These are fusion proteins in which the toxin has been replaced by a ribonuclease. Mammalian RNases are expected to be not immunogenic and not systemically toxic, as they are *pro*-toxins, which become toxic only upon their internalization in target cells mediated by the antibody moiety (21-23).

A fully human immunoRNase, denominated Erb-hRNase(Erbicin-human-RNase) (Figure 3), was constructed through the fusion of Erbicin with human pancreatic RNase (HP-RNase or RNase 1) (24).

The chimeric protein was found to retain the high binding affinity to ErbB2-positive cells of Erbicin and the enzymatic activity of native human pancreatic RNase. When tested in vitro on a series of malignant cells, Erb-hRNase was found to discriminate between target and non-target cells, and to specifically inhibit the proliferation of ErbB2-positive cells, with a stronger cytotoxicity on cells with a higher level of ErbB2.

Its antitumor activity has been also demonstrated in vivo on mice implanted with ErbB2-positive tumors (24,25).

The antitumor action of Erb-hRNase is dependent on the ability of this molecule to reach the cytosol and degrade RNA but it is somewhat limited by the finding (26) that the enzymatic activity of Erb-hRNase is inhibited by the RNase inhibitor (RI).

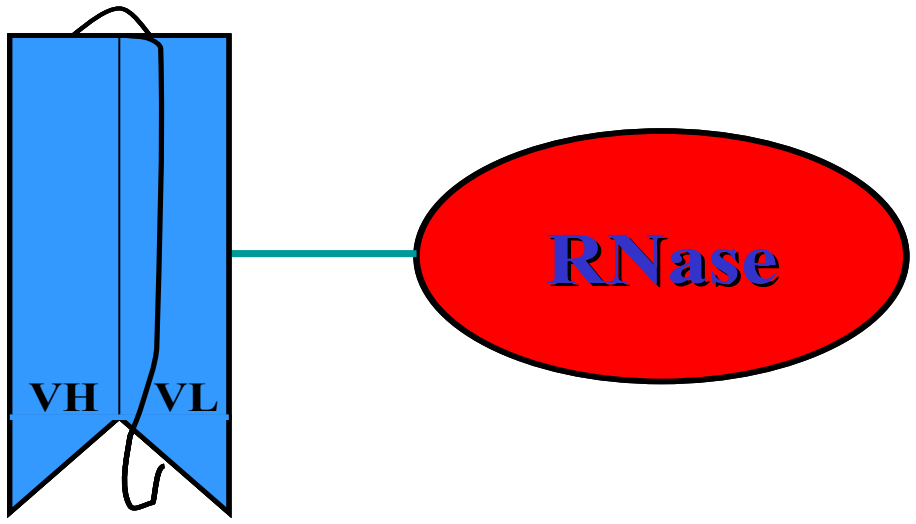

Figure 3 Schematic representation of the human immunoRNase **Erb-hRNase** (Erbicin-human-RNase).

In order to obtain a novel product, which is expected to be superior to the immunoagents currently used in the therapy of breast cancer, a novel immunoRNase has been designed, in which an Erbicin based scFv-Fc (Erb-hcAb, see above) replaces the scFv of Erb-hRNase (Figure. 4).

The latter should have the following advantages: the ability of inducing both ADCC and CDC, in addition to the RNase-based cytotoxicity; a prolonged half-life, due to its higher molecular size and the presence of the Fc; an increased avidity due to
the presence of two scFv moieties. Furthermore, this novel immunoagent could be resistant to the action of RI, due to the steric hindrance generated by the presence of the larger antibody moiety.

Here we report on the construction and characterization of a such novel fully human immunoRNase, made up of Erb-hcAb and HP-RNase, expressed in mammalian cell cultures.

This new immunoconjugate, called Erb-hcAb-RNase, has shown to fully retain the binding ability, ADCC and CDC properties of Erb-hcAb and to acquire the RNase activity, thus inhibiting tumor cell proliferation in vitro and in vivo more efficiently than the parental Erb-hcAb.

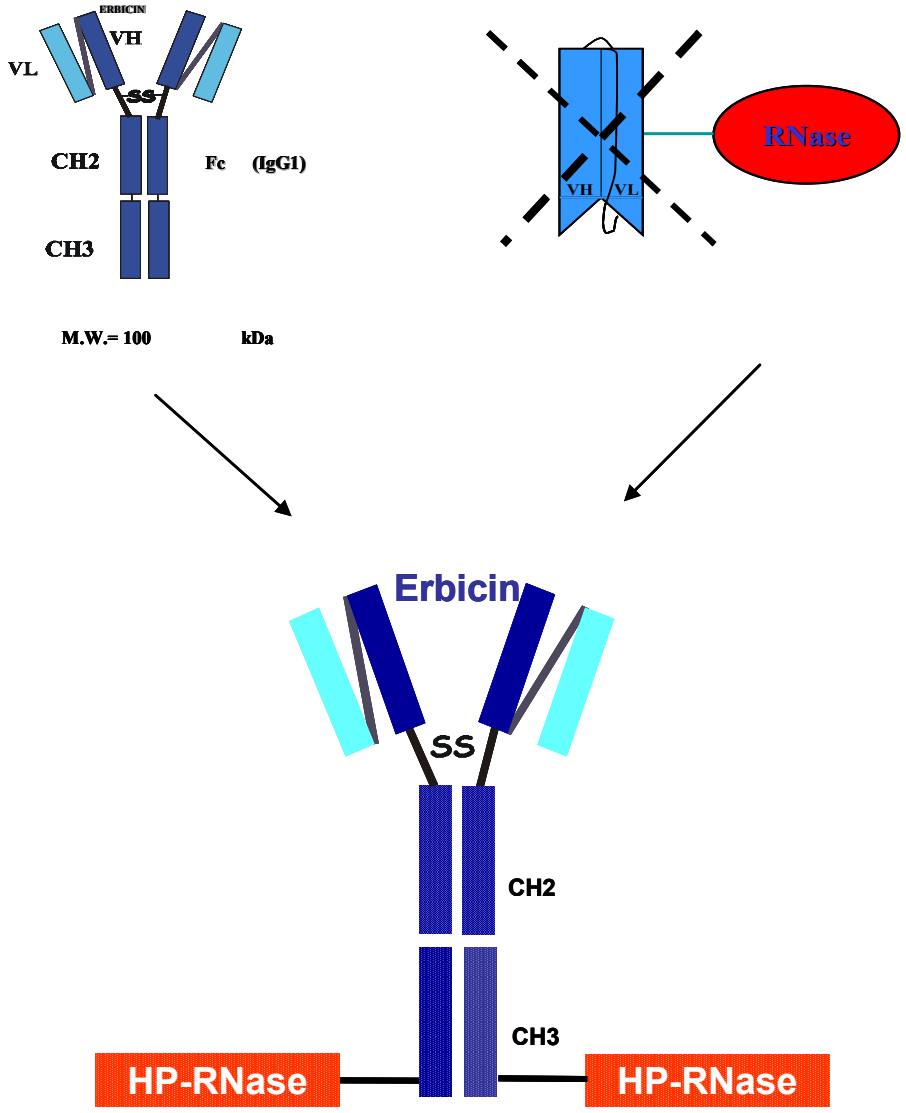

Figure 4. Design of a novel immunoRNase in which an Erbicin based scFv-Fc (Erb-hcAb) replaces the scFv of Erb-hRNase.

MATERIALS AND METHODS

2.1 *Cell cultures and antibodies*

The SKBR3 cell line from human breast cancer and the A431 cell line from human epidermoid carcinoma were cultured in RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK). The TUBO cell line from a BALB-neu T mouse-derived mammary lobular carcinoma (kindly provided by Dr G Forni, University of Turin, Italy) was grown in DMEM (Gibco BRL). The media were supplemented with 10% foetal bovine serum (20% for TUBO cells), 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (all from Gibco BRL).

The antibodies used were: Herceptin (Genentech, South San Francisco, CA, USA); affinity isolated IgGs from a rabbit anti-HP-RNase antiserum (from Igtech, Salerno, Italy); horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (Pierce, Rockford, IL, USA); HRP-conjugated goat anti-human affinity-isolated IgGs (Fc specific) (Sigma); Erb-hcAb was prepared as previously described (12).

2.2 Peripheral blood lymphocytes

Peripheral blood lymphocytes (PBL) were obtained from peripheral blood mononuclear cells (PBMC) isolated by centrifugation on Lymphoprep gradients (Axis Shield PoC AS, Oslo, Norway) from normal donor buffy coats obtained from the Blood Bank of the Medical School of the University of Naples 'Federico II'. After the separation, PBL were washed twice and incubated in RPMI 1640 medium (Gibco BRL) for 2 h at 37°C to remove adherent cells. The nonadherent cells were used as natural cytotoxic effectors without any additional treatment.

2.3 Construction and production of the anti-ErbB2 Erb-hcAb-RNase

The cDNA coding for the human compact antibody Erb-hcAb (12) was amplified from plasmid pIg1plus (R & D Systems, Minneapolis, USA) by PCR using as forward and reverse primers oligonucleotides containing at their 5' and 3' ends a *SalI* and a *Not*I site, respectively: 5'-ACGCGTCGACCAGGTGCAGCTGTTG-3';

5'-ATAAGAATGCGGCCGCTTTACCCGGAGACAGG-3'. The PCR fragment was then digested with *Sal*I and *Not*I (New England Biolabs, Hertfordshire, UK) for cloning into the eukaryotic pCMV-ER-myc expression vector (Invitrogen) downstream to the leader Endoplasmic Reticulum (ER) signal peptide sequence.

In order to obtain the chimeric construct, the gene fragment coding for the human pancreatic RNase was cloned downstream to the Erb-hcAb sequence by inserting a spacer encoding a 11-amino acid peptide linker (AAASGGPEGGS) between the scFv-Fc and the RNase. The cDNA coding for HP-RNase and the peptide spacer, previously generated (24) with NotI restriction sites at its 3' and 5' ends, was digested with *Not*I and cloned into the corresponding site of pCMV-ER-myc vector downstream to the sequence encoding the human Erb-hcAb.

The correct directional insertion of the RNase gene in the NotI sites was assessed by digestion with suitable endonucleases (New England Biolabs). Sequence analyses confirmed the expected DNA sequence.

The fusion protein was produced by transfecting 293 T (Human Embryonic Kidney) cells with the recombinant vector. In brief, cells grown in DMEM containing 10% FCS at 70–80% confluency were transfected with 5 μg of expression vector by using the Superfect reagent (Qiagen, Valencia, CA, USA). Stable transfectants were selected in the presence of G418 (Sigma) at a concentration of 1 mg ml^{-1}. The expression of the antibody construct was determined in the culture medium by quantitative ELISA. For recombinant protein production, transfected 293T cells were expanded to near confluence in selective DMEM medium containing 0,5 mg/ml G418, 4 mg/ml glucose, 10% foetal bovine serum, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and were then grown for 3–4 days in serum-free medium.

The recombinant fusion protein, henceforth termed Erb-hcAb-RNase, secreted by transfected 293T cells, was purified from culture medium by affinity chromatography on a protein A-Ceramic Hyper D®F column (Pall Corporation Port Washington, NY) loaded with 300–500 ml of conditioned medium, washed with 10 volumes of 100 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl, and 10 volumes of 10 mM Tris-HCl, pH 8.0. The protein eluate was obtained with 50 mM glycine pH 3.0, and immediately neutralised with 1/10 volume of 1 M Tris-HCl, pH 8.0.

2.4 RNase activity and inhibition

RNase activity was tested with the acid-insoluble RNA precipitation assay as described previously (28) on yeast RNA (8 mg/ml). RNase zymograms, carried out on SDS-PAGE electropherograms, were performed as described previously (26). For inhibition assays, appropriate amounts of Erb-hcAb-RNase and Erb-hRNasewere pre-incubated with increasing concentrations of cRI at 37° C for 10 min before starting the activity test described above. Ribonuclease inhibitor was purchased from Promega (Madison, WI, USA); its concentration was determined as previously described (30).

2.5 ELISA assays

ErbB2-positive SKBR3 cells and ErbB2-negative A431 control cells, harvested in nonenzymatic dissociation solution (Sigma), were washed and transferred to U-bottom microtitre plates (1 $x10^5$ cells per well). After blocking with PBS containing 6% bovine serum albumin (BSA), cells were incubated with conditioned medium or purified immunoagents in ELISA buffer (PBS/BSA 3%) for 90 min. The pelleted cells were washed, resuspended in 100 μl of ELISA buffer, and incubated with an anti-human IgG (Fc-specific) mAb (Sigma) for detection of Erb-hcAb and Erb-hcAb-RNase. The latter was detected also with an anti-HP-RNase IgG antibody followed by HRP-conjugated goat anti-rabbit immunoglobulin. After 1 h, the plates were centrifuged, washed with ELISA buffer, and reacted with 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma). Binding values were determined from the absorbance at 450 nm, and reported as the mean of at least three determinations (s.d. $\leq$5%).

2.6 Cell growth inhibition assays

Cells were seeded in 96-well, flat-bottom plates; SKBR3 cells at a density of 1.5 $x10^4$ well; A431 at a density of 5 $x10^3$ well. After incubation at 37° for 72h with the protein under test, viable cells were counted by the Trypan blue-exclusion test. Cell viability was determined in triplicate by using methyl tetrazolium (MTT) test (Sigma) according to the manufacturer's recommendations. The resulting absorbance at 570 nm was measured in a

microplate counter (Multilabel Counter Victor 3, Perkin Elmer). 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. Typically, standard deviations were below 5%.

2.7 ADCC and CDC tests

Target and control cells were detached from culture dishes with a cell dissociation solution (Sigma) and transferred to round-bottom 96-well plates (2×10^4 cells per well). For ADCC assays, target or control cells were treated with the immunoagents (5 μg ml^{-1} of serum-free medium) and peripheral blood lymphocytes (PBL) at 37°C for 3–4 h. For CDC assays, cells were incubated at 37°C with human serum. Cultures were performed in triplicate in a final volume of 200 μl. Controls included target cells incubated in the absence of effector cells, or in the presence of either serum or immunoagent alone. Tumour cell lysis was determined by measuring the release of lactate dehydrogenase (LDH) using a LDH detection kit (Roche, Mannheim, Germany). ADCC or CDC was calculated as the percent of cytolysis measured in the presence of immunoagent and PBL or human serum, for ADCC and CDC, respectively, taking as 100% the maximal LDH release determined by lysis of target cells with 1% Triton X-100.

2.8 In vivo antitumour activity

All experiments were performed with 6-week-old female Balb/cAnNCrlBR mice (Charles River Laboratories, Calco, Italy). TUBO cells (5×10^5) were suspended in 0.2 ml sterile PBS and injected subcutaneously (day 0) in the right paw. At day 7, when tumours started to appear, the mice were divided into three groups. At day 15, when tumours were clearly detectable, Erb-hcAb-RNase dissolved in PBS was administered i.p. at doses of 1.8 mg kg^{-1} of body weight for 7 times at 72 h intervals. The second group of animals was treated with equimolar doses (1.3 mg kg^{-1} of body weight) of Erb-hcAb, dissolved in PBS, and administered i.p. for 7 times at 72 h intervals. The third group of control animals was treated with identical volumes of sterile PBS. During the period of treatment, tumour volumes (V) were measured with caliper and calculated by the formula of rotational ellipsoid $V=A \times B^2/2$ (A is the axial diameter, B the rotational

diameter). All mice were maintained at the animal facility of the Department of Cellular and Molecular Biology and Pathology, University of Naples "Federico II". Animal studies were conducted in accordance with the Italian regulation for experimentation on animals.

All *in vivo* experiments were carried out with ethical committee approval and met the standards required by the UKCCCR guidelines (28).

RESULTS

3.1 Production and purification of a novel fully human anti-ErbB2 compact antibody-RNase conjugate

A new human anti-ErbB2 immunoagent was generated by fusing human pancreatic RNase (HP-RNase) with the fully human anti-ErbB2 compact antibody (Erb-hcAb) (12). The cDNAs coding for the human compact antibody Erb-hcAb and human pancreatic RNase were amplified by PCR and cloned into the eukaryotic pCMV-ER-myc expression vector. In particular, the cDNA encoding human pancreatic RNase was cloned downstream to the sequence encoding the carboxy terminus of the scFv-Fc (Erb-hcAb) by adding a spacer encoding a 11-amino acid residue peptide linker (*AAASGGPEGGS*) to minimize the steric hindrance between the two moieties of the chimeric protein (figure 5). The recombinant plasmid, sequenced to confirm faithful cloning, was stably transfected in 293 T (Human Embryonic Kidney) cells, and the recombinant construct was expressed as a secretion product into the culture medium. Once selected by quantitative ELISA assays, the clone producing the highest levels of Erb-hcAb-RNase was used for the production of the chimeric immunoagent, which was then purified by affinity chromatography (see Methods). The immunoagent was named Erb-hcAb-RNase (anti-ErbB2 human compact antibody-RNase).

3.2 Characterization of Erb-hcAb-RNase

When Erb-hcAb-RNase was analysed by SDS–PAGE (figure 6), it was found to migrate under reducing conditions with the expected molecular size of about 70 kDa (figure. 6B), and as a dimer of about 140 kDa under nonreducing conditions (figure. 6C). This result indicates that the fusion protein is expressed as a disulphide-linked dimer. Western blotting analyses performed with either an anti-human Fc or an anti-HP-RNase antibody demonstrated immunoreactivity of the purified, dimeric protein with a molecular size of 140 kDa (figure 6 D-E).

The fusion protein was then tested for enzymatic activity by a zymogram, developed using yeast RNA as a substrate. As illustrated in

figure. 6F, a single active band was detectable, corresponding to the size of Erb-hcAb-RNase.

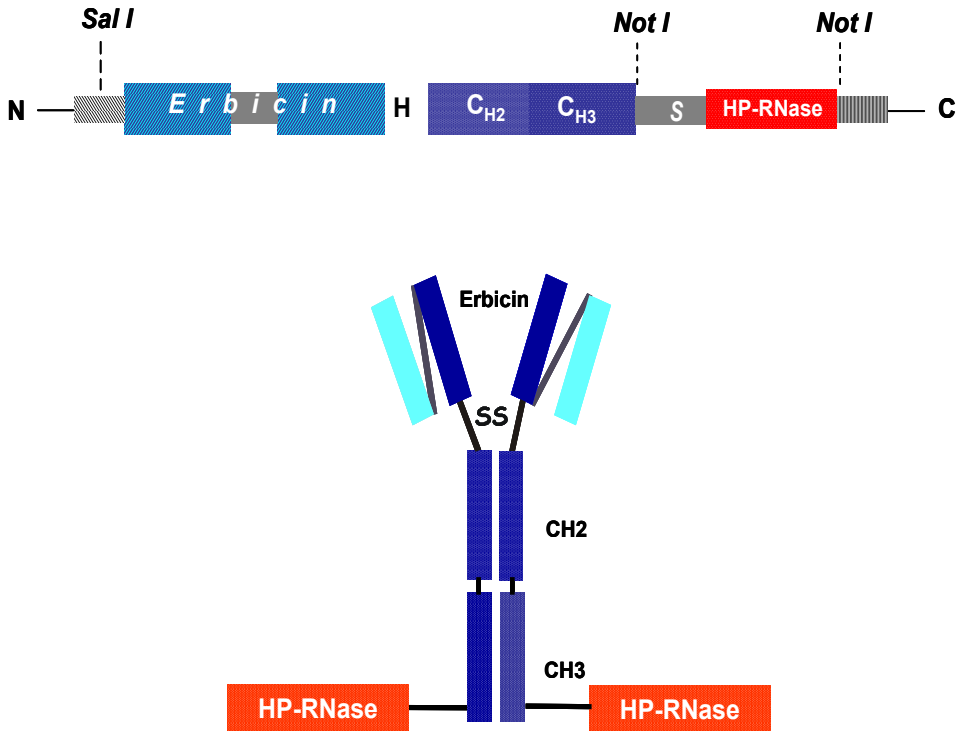

Figure 5 Schematic representation of the chimeric ImmunoRNase, Erb-hcAb-RNase, obtained by fusing the human compact antibody Erb-hcAb, and the human pancreatic RNase. **Erbicin,** the human anti-ErbB2 scFv; **H**, hinge; **CH2-CH3**, the heavy constant domains of the human IgG1 Fc; **S**, the spacer peptide AAASGGPEGGS linking the scFv-Fc and the RNase moieties; **HP-RNase**, the RNase moiety

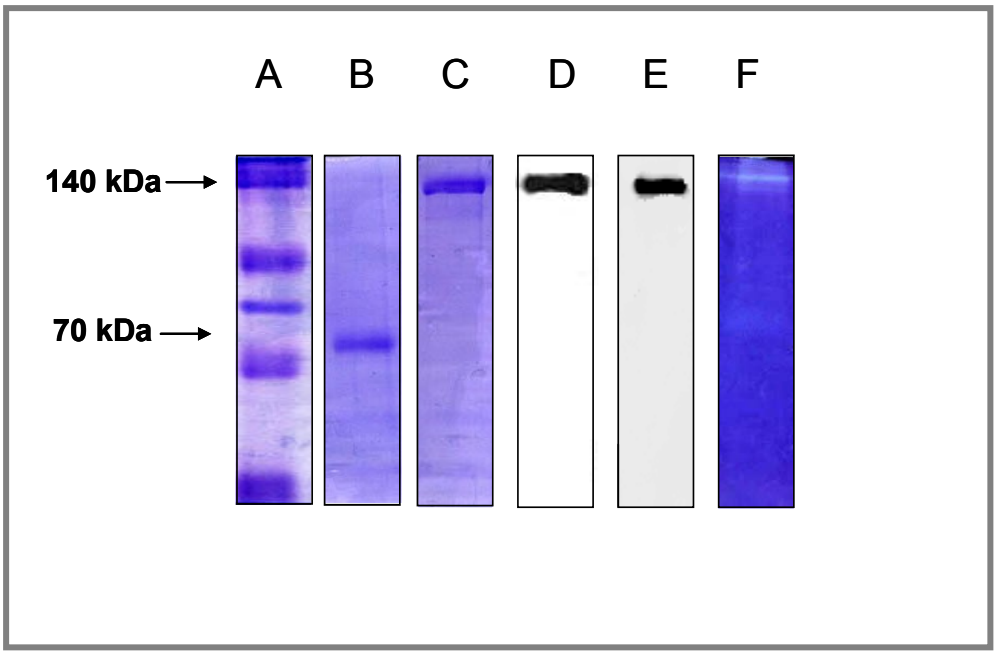

Figure 6. SDS–PAGE and Western blotting analyses of purified Erb-hcAb-RNase. Erb-hcAb-RNase was run under reducing (lane B) or nonreducing (lane C) conditions; molecular weight standards are in lane A; Western blotting analyses of the purifed sample with an anti-human IgG1 (Fc specific) (lane D) or with the anti-human pancreatic RNase antibody (lane E); Zymogram of Erb-hcAb-RNase using yeast RNA as a substrate in lane F.

In a parallel assay the first generation anti-ErbB2 immunoRNase (Erb-hRNase), made up of the Erbicin scFv and HP-RNase, was used as a positive control (data not shown).

The ribonucleolytic activity of the purified Erb-hcAb-RNase was further tested with the acid-insoluble RNA precipitation assay (28) , by which the chimeric immunoagent was found to have a specific activity of 730 ± 25 units/nmol. This result was confirmed for several preparations of the recombinant fusion protein. Because the specific activity of hErb-hRNase, i.e. the monomeric anti-ErbB2 immunoRNase, previously tested, is 950 ± 25 units/nmol (24), we can conclude that Erb-hcAb-RNase retains about 80% of the activity of the first generation ImmunoRNase (figure 7) .

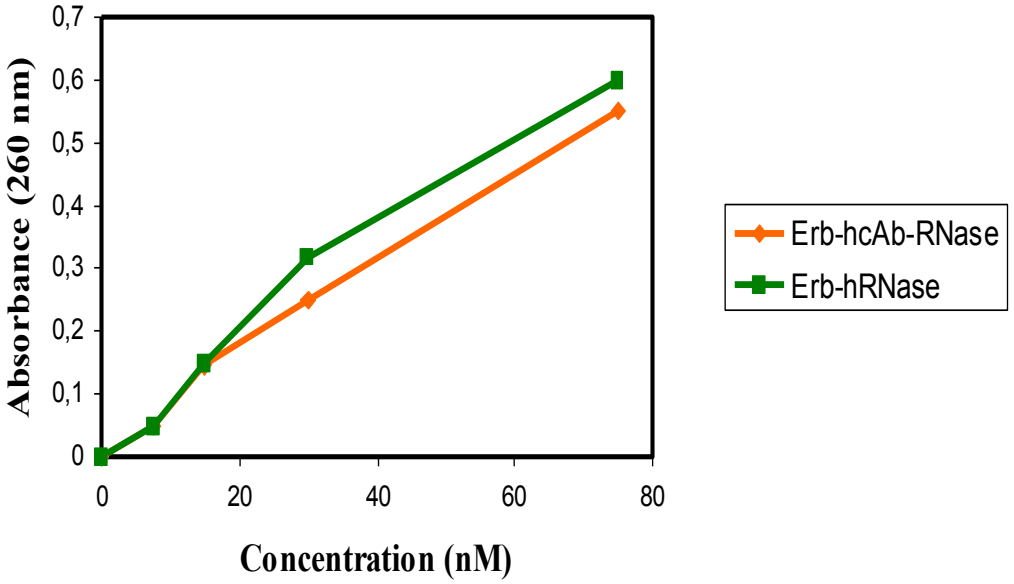

Figure 7 Enzymatic activity of the immunoRNases (IR): the anti- ErbB2 Erb-hcAb-RNase (orange) or ERB-hRNase (green)

To determine the sensitivity of Erb-hcAb-RNase to RI inhibition, the enzymatic assays were repeated in the absence or in the presence of increasing concentrations of RI. Erb-hRNase was used as a positive control, as it was previously found to be fully inhibited by RI (27).

As shown in figure 8, the novel chimeric protein was found to be susceptible of inhibition by RI even though it was found to be less sensitive then the monomeric immunoRNase. Indeed, the new ImmunoRNase retains a residual 30% of the enzymatic activity even at a cRI/IR ratio of about 5, whereas the activity of the monomeric IR is completely inhibited at a cRI/IR ratio of about 1 (figure 8).

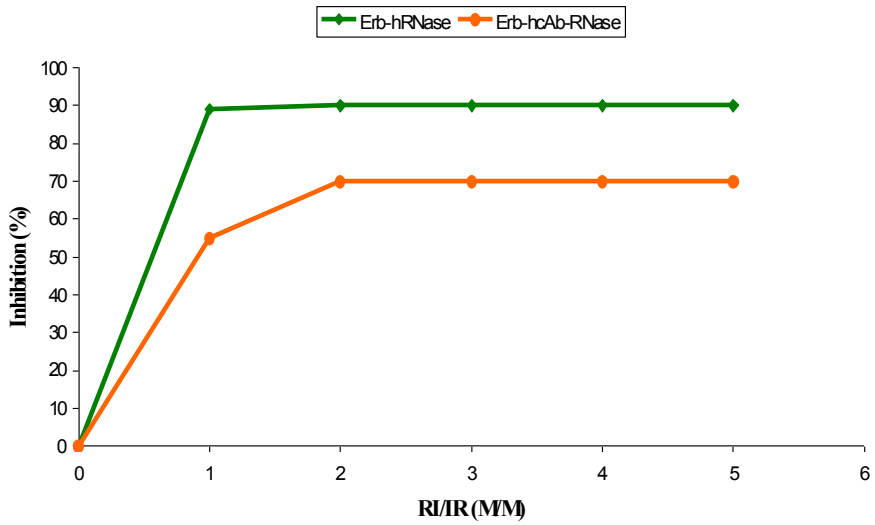

Figure 8. Effects of the RNase inhibitor (RI) on the enzymatic activity of the immunoRNases (IR).
Inhibition by RI of the catalytic activity of the anti- ErbB2 Erb-hcAb-RNase (circles) or ERB-hRNase (rhomboids) was measured at increasing RI/IR ratios

This result could be considered not surprising as the larger molecular size of the antibody fragment of Erb-hcAb-RNase with respect to that of the scFv in Erb-hRNase could hinder the interactions between RI and the RNase.

When the ability of the recombinant fusion protein to bind to ErbB2-positive cells was analysed by ELISA assays (figure 9), Erb-hcAb-RNase was found to fully retain the specificity and the affinity of the parental compact antibody for mammary carcinoma ErbB2-overexpressing SKBR3 cells. As a negative control, we used A431 cells (from human epidermoid carcinoma), which express very low levels of ErbB2. The apparent binding affinity of Erb-hcAb-RNase for the ErbB2 receptor, that is the concentration corresponding to half-maximal saturation, was about 1 nM, identical to that of the parental Erb-hcAb.

These results demonstrate that the compact antibody and the RNase retain their biological functions in the chimeric protein.

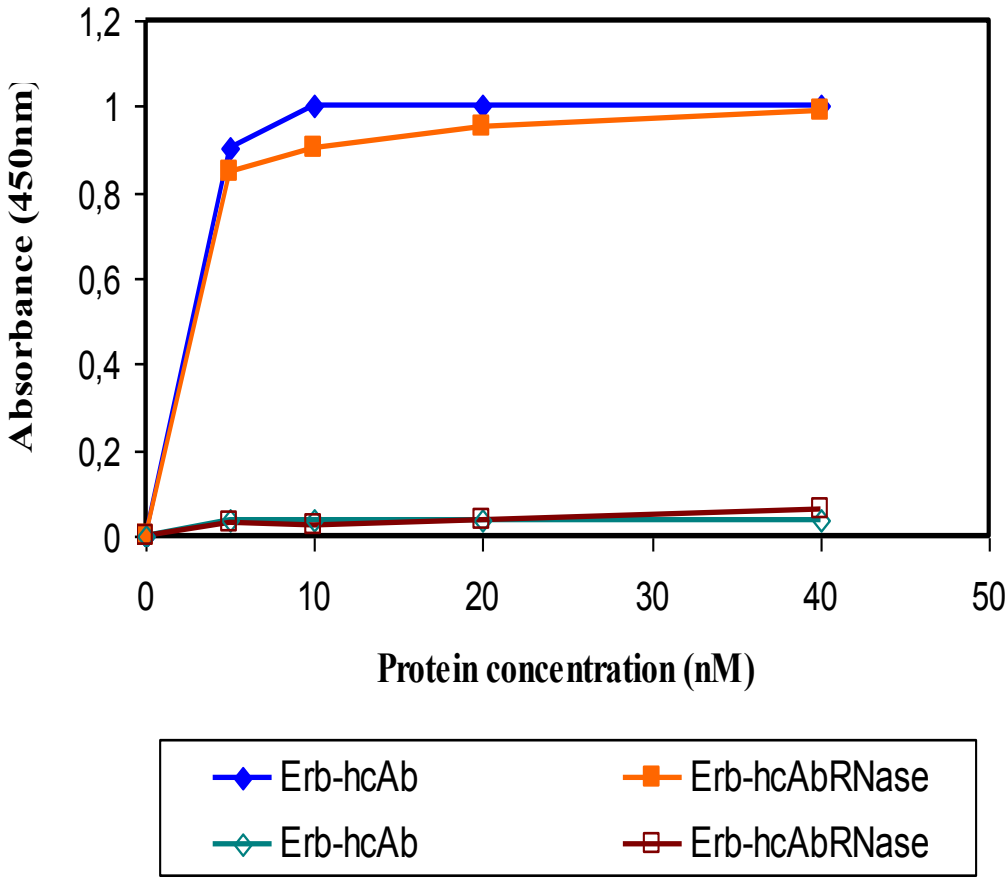

Figure 9. Binding curves of Erb-hcAb-RNase or ERB-hcAb to ErbB2-positive (SKBR3) and -negative (A431) cell lines. SKBR3 cells were tested by enzyme-linked immunoasorbent assay with Erb-hcAb-RNase (orange squares), or with Erb-hcAb (blue rhomboids), used as a control; A431 cells were tested as indicated above with Erb-hcAb-RNase or Erb-hcAb (empty squares and rhomboids, respectively).

3.3 Biological effects of Erb-hcAb-RNase on ErbB2-positive tumour cells

To assess the *in vitro* effects of Erb-hcAb-RNase on tumour cell growth, the ErbB2-positive SKBR3 and the ErbB2-negative A431 cell lines were incubated with increasing concentrations of Erb-hcAb-RNase or ErbB-hcAb, used as a control. As shown in figure 10, Erb-hcAb-RNase inhibited the growth of SKBR3 cells in a dose-dependent manner, showing an antiproliferative effect more potent than that observed for the parental Erb-hcAb. The immunoagent did not have any effect on the proliferation of ErbB2-negative A431 cells (see figure 10).

To investigate whether Erb-hcAb-RNase was capable of recruiting immune effector functions *in vitro*, assays for cytolysis of tumour cells as induced by PBL, or complement, were performed. To determine the capacity of Erb-hcAb-RNase to selectively trigger ADCC towards antigen-expressing cells, SKBR3 and A431 cells were incubated for 3 h with increasing amounts of effector PBL in the absence or in the presence of Erb-hcAb-RNase ($5\,\mu g\,ml^{-1}$).
We used as positive controls both Erb-hcAb and Herceptin. As shown in figure 11, Erb-hcAb-RNase lysed SKBR3 target cells in the presence of PBL with an efficacy comparable to that of the parental Erb-hcAb.
The extent of lysis reached almost 80% of treated cells at a ratio of 100:1 (effector to target cells), whereas Herceptin induced about 65% lysis at the same ratio.
No effects were detected in parallel assays carried out with ErbB2-negative A431 cells (data not shown), thus demonstrating the specificity of the Erb-hcAb-RNase-dependent cell-mediated cytolytic activity.

Thus, we can conclude that the presence of the RNase in the new construct does not affect the interactions between the Fc region of Erb-hcAb and the CD16 receptor of the natural killer cells.

To test the ability of Erb-hcAb-RNase of inducing CDC against ErbB2-positive tumour cells, SKBR3 target cells were incubated for 6 h with Erb-hcAb-RNase (10 or $30\,\mu g\,ml^{-1}$) in the absence or in the presence of human serum as a source of complement. As illustrated in Figure 12, Erb-hcAb-RNase was found to effectively lyse SKBR3 cells in the presence of serum in a similar fashion of Erb-hcAb, with an average specific lysis of 40% after 6 h.

CDC was not detected when ErbB2-negative A431 cells were incubated with Erb-hcAb-RNase and human serum (data not shown).

Similarly, as previously reported (12, 32), no lysis was detectable when SKBR3 cells were treated with Herceptin in the presence of human serum (see figure 12).

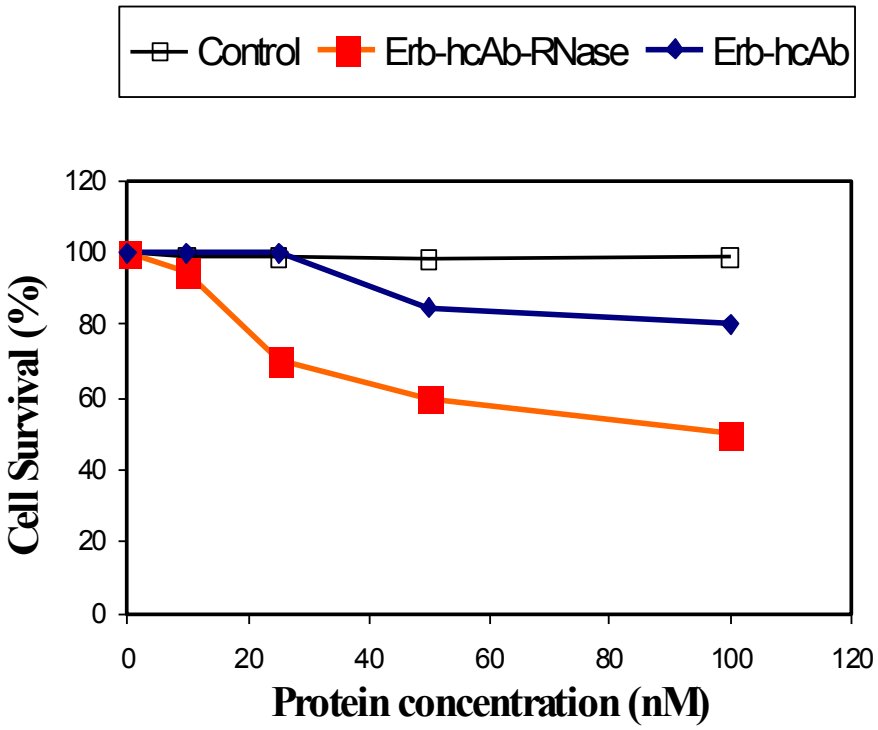

Figure 10. *In vitro* effects of Erb-hcAb-RNase on cell survival. Dose response curves of ErbB2-positive SKBR3 (fully symbols), and ErbB2-negative A431 cells (empty symbols), treated for 72 hours with Erb-hcAb-RNase (squares).
The effects of Erb-hcAb (rhomboids), used as a positive control, are also shown.

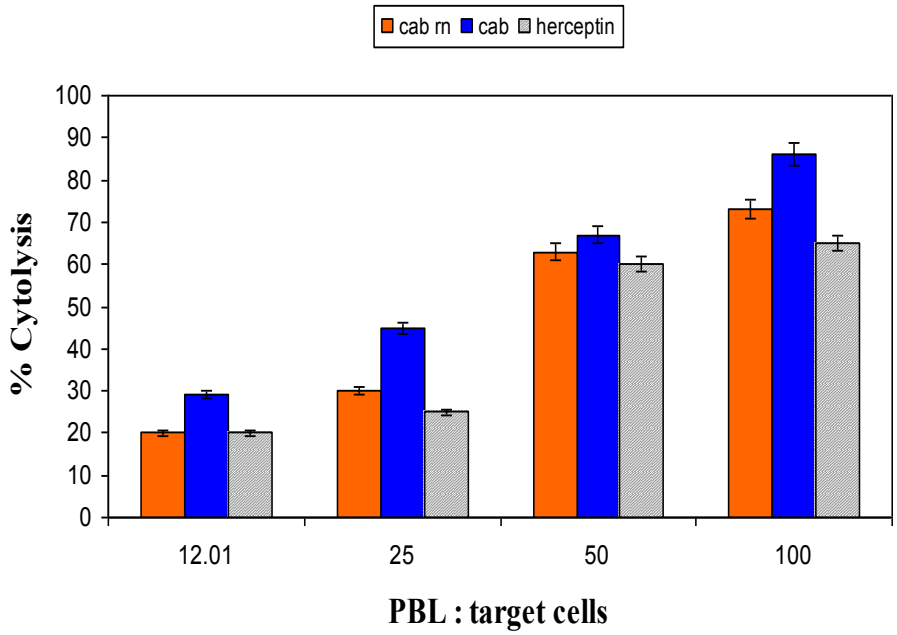

Figure 11 Antibody-dependent cytotoxicity assays of Erb-hcAb-RNase. SKBR3 cells treated with PBL as effector cells at four different ratios in the presence of Erb-hcAb-RNase (orange bars), Erb-hcAb (blue bars) or Herceptin (striped bars) used as positive controls.

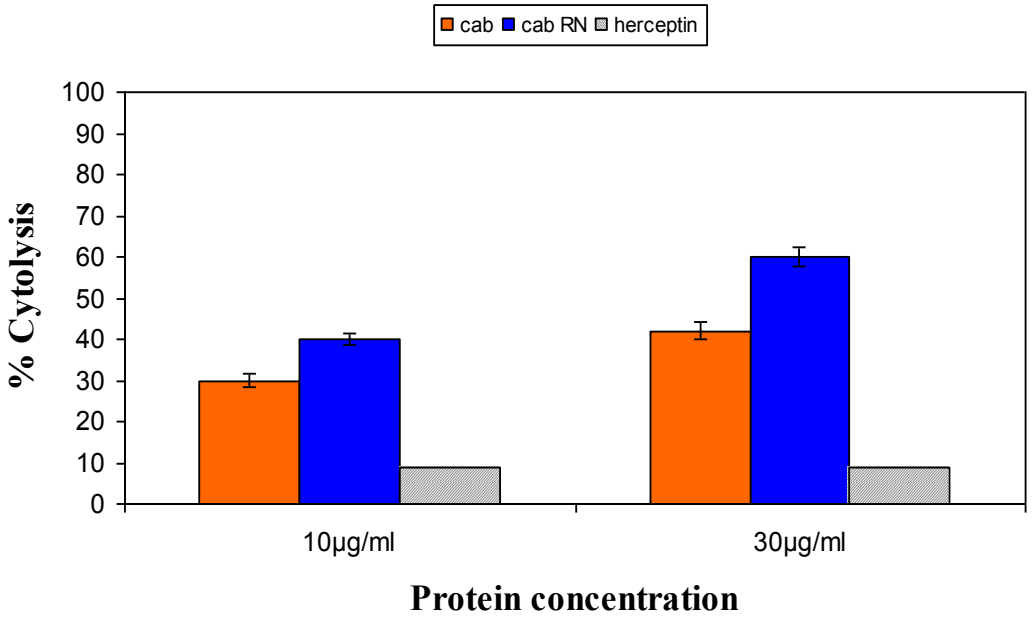

Figure 12. Complement-dependent cytotoxicity assays of Erb-hcAbRNase.
SKBR3 cells were incubated for 6h in the presence of human serum as a source of complement with Erb-hcAb-RNase (orange bars), Erb-hcAb (blue bars), used as a positive control, or Herceptin (striped bars), used as a negative control, at concentrations of 10µg/ml^{-1}and 30µg/ml^{-1}.

3.4 In vivo antitumor effects of Erb-hcAb-RNase

For *in vivo* studies Erb-hcAb-RNase was tested on murine TUBO tumour cells expressing ErbB2 of rat origin (33).
When administered to female mice, TUBO cells induce tumours very similar to the alveolar-type human lobular mammary carcinomas (34).

In order to compare the *in vivo* antitumour efficacy of this novel immunoagent with that of the parental Erb-hcAb, the effects of equimolar doses of Erb-hcAb and Erb-hcAb-RNase were tested in parallel on the same experimental model.
In particular, to detect the differential potency of the two immunoagents, they were administered at lower doses than those used for peritumoral administrations of Erb-hcAb in the previous experiment (13). when a dramatic reduction (96%) in tumor volume was observed.
As shown in figure 13, the treatment of mice bearing TUBO tumours with seven doses, at 72 h intervals, of 1.8 mg kg^{-1} of Erb-hcAb-RNase induced a significant reduction (about 50 %) in tumour volume and showed more potent antitumor effects than those observed for the parental Erb-hcAb (doses of 1.3 mg kg^{-1}).
During the period of treatment, the animals did not show signs of wasting or other visible signs of toxicity.

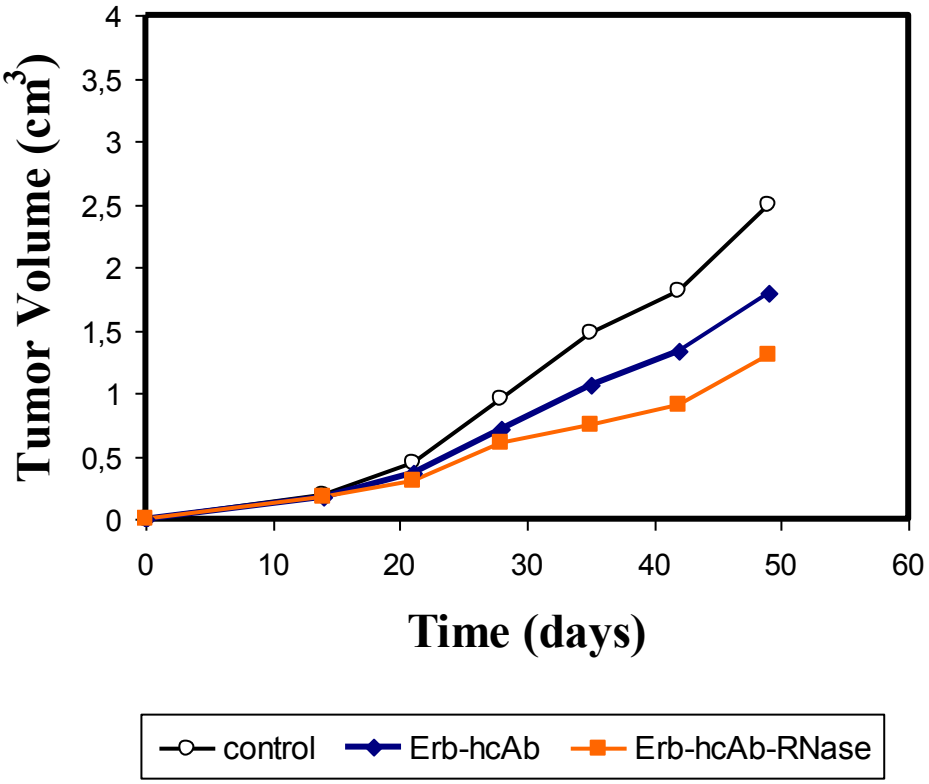

Figure 13. *In vivo* antitumor effects of Erb-hcAb-RNase.
Tumor growth was followed in mice inoculated s.c. with $5x10^5$ TUBO mammary carcinoma cells. Control animals (empty circles) were treated with sterile PBS solution.
Treated animals were injected with Erb-hcAb-RNase (orange squares), starting at day 14. Seven doses, each of 1.8 mg kg-1 of body weight, were administered at 72 h intervals i.p.
In a parallel experiment Erb-hcAb (blue rhomboids) was administered at equimolar doses (1.3 mg/kg $^{-1}$) as indicated for Erb-hcAb-RNase, for comparison.

DISCUSSION/CONCLUSIONS

Immunotoxins (ITs), based on toxins fused to antibody moieties specifically reactive to a certain type of tumor cells, have been designed for a novel approach in anticancer therapy. However, the non-specific toxicity of ITs associated to vascular leak syndrome and/or hepatotoxicity, as well as the immunogenicity of their bacterial or plant toxins have often limited the outcome of immunotoxins as anticancer drugs (18-20).

To circumvent these problems immunoRNases (IRs), based on the immunotoxin principle, have been proposed (21-23). In the immunoRNases, the toxin moiety of immunotoxins is replaced by a non-toxic and non-immunogenic RNase, which becomes toxic only upon its internalization mediated by the antibody moiety in the target cells.

We previously reported on a fully human immunoRNase obtained by fusing a human single-chain antibody fragment (scFv) and a human RNase (24), named Erb-hRNase, which is directed to the ErbB2 receptor, overexpressed in many carcinomas, especially in breast carcinoma (6,7).

Erb-hRNase was found to exert a powerful and selective antitumor action *in vitro* and *in vivo*. However, its successful therapeutic use could be somewhat limited by the lack of antibody effector functions, as well as by its monovalent nature and by its small size (less than 50 kDa), which could be responsible for a reduced tumor retention and a faster clearance from the blood circulation, respectively.

In this study, we report on the production and characterization of a novel second generation fully human IR targeting the ErbB2 receptor, called Erb-hcAb-RNase.

It results from the fusion of the fully human anti-ErbB2 compact antibody Erb-hcAb (12) with HP-RNase. We have shown here that both the antibody and RNase moieties preserve their biological actions in the immunoconjugate. This is endowed with the following properties:

1. it recognises one of the most specific tumour-associated antigens, such as ErbB2, with an affinity comparable to that of the parental compact antibody;
2. it retains the enzymatic activity of the first generation immunoRNase (Erb-hRNase) but, at difference with the monovalent IR, it is only partially susceptible to RI inhibition;
3. it displays effective antibody effector functions with an efficacy comparable to that of the parental Erb-hcAb;
4. it inhibits efficiently the proliferation of ErbB2-positive tumor cells both *in vitro* and *in vivo* with antitumor effects more potent than those observed for the parental compact antibody. The latter results can be explained by the additional toxic action of the internalized RNase;
5. the size of Erb-hcAb-RNase should be better suited for therapeutic applications due to the potential prolonged half life with respect to the first generation scFv-based IR and to better penetration properties than full size IgG-toxin immunoconjugates.

It has been already reported by Menzel and colleagues on a powerful anti-CD30 scFv-Fc-RNase, made up of a CD30 lymphoma specific human scFv-Fc fused to human pancreatic RNase, showing specific binding and in vitro cytotoxicity on CD30+ lymphoma cells at nM concentrations (24). However this IR was not tested for its effector functions (ADCC and CDC) and for its *in vivo* antitumor activity.

To our knowledge, Erb-hcAb-RNase is to date the first fully human antibody-RNase to be constructed and tested with satisfactory results *in vivo* demonstrating for the first time that the presence of the RNase does not hinder the antibody effector functions (ADCC and CDC).

On the basis of the effectiveness and selectivity of its antitumor action on target cells, Erb-hcAb-RNase represents a promising valuable tool in cancer therapy, thus supporting the hypothesis that the scFv-Fc-RNase format is the most appropriate for the production of a novel generation of IR better suited for therapeutic applications, as it combines the advantages of the first generation IR with those of functional relevant antibody domains.

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