Int. J. Cancer: **73,** 117–124 (1997)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

TARGETED THERAPY OF SCHWANNOMA CELLS IN IMMUNOCOMPETENT RATS WITH AN erbB2-SPECIFIC ANTIBODY-TOXIN

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Over-expression of the erbB2-receptor tyrosine kinase is frequently observed in many human tumors of epithelial origin. Due to its causal involvement in malignant transformation and its presence on the tumor cell surface erbB2 is an attractive target for directed tumor therapy. We earlier described the potent anti-tumoral activity of the recombinant single-chain antibody toxin scFv(FRP5)-ETA *in vitro* and in nude mouse tumor models *in vivo.* This molecule consists of the variable domains of the heavy and light chains of an erbB2-specific antibody genetically fused to a truncated *Pseudomonas* exotoxin A. Here we have investigated the *in vivo* effects of this immunotoxin on erbB2 expressing NV2Cd schwannoma cells growing as s.c. tumors in syngeneic BDIX rats. Established tumors were treated either locally by intratumoral injection of scFv(FRP5)-ETA or systemically by injection into the tail vein. Both routes of application resulted in pronounced inhibition of tumor growth with local treatment being more effective. Treatment with 25 μg/day of scFv(FRP5)-ETA for 10 days suppressed tumor growth almost completely. Antibodies directed mainly against the toxin domain of the fusion protein developed in all animals treated. *Int. J. Cancer* 73:117–124, 1997.
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The *erb*B-2 gene encodes a 185-kDa transmembrane glycoprotein that is a member of the type I/erbB family of receptor tyrosine kinases which also includes epidermal-growth-factor (EGF) receptor, erbB3 and erbB4 (Peles and Yarden, 1993). Over-expression of erbB2 is frequently observed in human tumors arising at many sites, including the breast and the ovaries, where it correlates with an unfavorable prognosis (Hynes and Stern, 1994). Due to its role in cancer development and its accessible location on the cell surface erbB2 has been suggested as a promising target for directed therapy.

Pseudomonas aeruginosa exotoxin A is a well-characterized protein of 66 kDa which harbors the different activities required for cell binding, uptake into cells, and toxic activity in distinct functional domains (Allured *et al.,* 1986; Hwang *et al.,* 1987). Upon binding of the N-terminal domain I to target cells and internalization via receptor-mediated endocytosis the internal domain II becomes activated by proteolytic cleavage and facilitates the translocation of a C-terminal fragment containing the enzymatic domain III into the cytosol (Ogata *et al.,* 1992). There the toxin ADP-ribosylates elongation factor 2 which results in the inhibition of protein synthesis. On the basis of earlier studies demonstrating the utility of recombinant ETA as a potential therapeutic effector (Pastan and FitzGerald, 1991), we have already described chimeric ETA fusion proteins targeted to the different members of the erbB receptor family.

As a cell recognition domain these hybrid molecules employ either growth factors like the erbB3/erbB4 ligand Heregulin β1 (Jeschke *et al.,* 1995; Groner *et al.,* 1997), or recombinant single-chain antibody (scFv) fragments directed to erbB2 (Wels *et al.,* 1992, 1995) and the EGF receptor (Wels *et al.,* 1995; Schmidt *et al.,* 1997). ScFv molecules consist of the variable domains of the heavy and light chains of immunoglobulins connected by a flexible linker sequence (Winter and Milstein, 1991). The heterologous binding domains were genetically fused to a modified form of ETA which lacks the natural cell-binding domain of the toxin (Wels *et al.,* 1992). The resulting chimeric toxins were expressed in *E. coli* as recombinant molecules and their biological activities were characterized. High selectivity for cells expressing the respective target receptor and potent anti-tumoral activity were observed (Wels *et al.,* 1992, 1995; Groner *et al.,* 1997).

To evaluate the *in vivo* effects of such reagents, pre-clinical animal model systems are of fundamental importance. Since most of these molecules are specific for human tumor antigens, experimental settings chosen are often based on human tumor xenografts growing in immunocompromised animals. However, a major limitation of such animal models is their greatly impaired immunological response to the effector which is applied for therapy. Since recombinant toxins contain a large protein domain of bacterial origin, it is predictable that in an immunocompetent organism a humoral immune response directed against this type of therapeutics will develop which might neutralize the anti-tumoral activity.

Here we have evaluated the *in vivo* effects of the erbB2-specific scFv-*Pseudomonas* exotoxin-A fusion protein scFv(FRP5)-ETA in immunocompetent rats. Our tumor model is based on NV2Cd rat schwannoma cells stably transfected with a human *erb*B-2 cDNA construct (Nikitin *et al.,* 1996). Upon s.c. injection NV2Cd-erbB2 cells formed rapidly growing tumors in syngeneic BDIX rats. Treatment of established tumors with the erbB2-specific antibody toxin by intratumoral application resulted in pronounced inhibition of tumor growth. Systemic treatment also led to growth inhibition, but to a lesser extent. During the course of the treatment all animals developed a strong antibody response against the fusion protein.

MATERIAL AND METHODS

Cells and culture conditions

The SKBR3 and MDA-MB468 human breast-tumor cell lines and the A431 human epidermoid-tumor cell line were maintained in DMEM (GIBCO, Eggenstein, Germany) supplemented with 10% heat-inactivated FBS. The established rat schwannoma cell line NV2Cd and its derivative NV2Cd-erbB2 stably transfected with a human *erb*B-2 cDNA construct (kindly provided by Dr. M. Rajewsky) were generated by Nikitin *et al.* (1996). The cells were maintained in DMEM supplemented with 8% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.5 mg/ml G418 (GIBCO) for NV2Cd-erbB2.

Bacterial expression of scFv(FRP5)-ETA and preparation of inclusion bodies

From the plasmid pSW202-5 (Wels *et al.,* 1995), which encodes under the control of the IPTG-inducible tac promoter, the erbB2-

Abbreviations: ETA, *Pseudomonas* exotoxin A; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; IC_{50}, 50% inhibitory concentration; IPTG, isopropyl-β-D-thiogalactopyranoside; scFv, single chain Fv antibody fragment; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidenedifluoride.

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: SFB 364 C1.

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Received 2 March 1997; Revised 6 June 1997

specific scFv(FRP5)-ETA immunotoxin fused at the N-terminus to the *E. coli* ompA signal peptide, a synthetic FLAG epitope, and a polyhistidine tag, the ompA signal sequence was removed, resulting in plasmid pSW220-5 which upon induction of expression allows the accumulation of large amounts of recombinant protein as inclusion bodies. Plasmid pSW220-5 was transformed into *E. coli* HB101 (Bolivar and Backman, 1979). A single colony was grown overnight at 37°C in terrific broth (12 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 1.7 mM KH_2PO_4, 7.2 mM K_2HPO_4) containing 0.6% glucose and 100 µg/ml ampicillin. The culture was diluted 30-fold in the same medium and grown at 37°C to an OD_{550} of 10; expression of the recombinant protein was induced with 1% lactose for 16 hr at 37°C. Cells were harvested by centrifugation at 10,000*g* for 10 min at 4°C and the cell pellet from 1 l of culture was re-suspended in 2 × v/w of ice-cold 40 mM Tris, pH 8.0, at 0°C. Cells were disrupted in a French press and the lysate was diluted 1:4 in ice-cold 40 mM Tris, pH 8.0 and centrifuged for 20 min at 9,000*g*. The supernatant was discarded, and the pellet was washed with 200 ml of ice-cold 40 mM Tris, pH 8.0, and centrifuged as described above. The washed pellet containing the inclusion bodies was stored at −70°C.

Purification of scFv(FRP5)-ETA

Inclusion bodies were re-suspended in 10 × v/w of buffer A (8 M urea, 50 mM Tris, pH 9.0, 0.5 M NaCl) for 30 min at room temperature. The suspension was clarified by centrifugation at 16,000*g* for 30 min at 4°C and solubilized scFv(FRP5)-ETA protein was purified via binding of the polyhistidine tag included in the molecule to Ni^{2+} saturated chelating sepharose (Pharmacia Biotech, Freiburg, Germany) equilibrated with buffer A. The solution was loaded onto the column at a flow rate of 1.5 ml/min. Unspecifically bound proteins were removed by washing with buffer A containing 20 mM and 40 mM imidazole. Specifically bound scFv(FRP5)-ETA protein was eluted with buffer A containing 200 mM imidazole. The protein content of the eluate was determined using a protein quantification kit (Biorad). The typical yield of purified protein was 30 mg/l of original bacterial culture with a purity of more than 90%, as determined by SDS-PAGE and Coomassie-brilliant-blue staining. ScFv(FRP5)-ETA was renatured by rapid 1:10 dilution of the purified protein into a buffer containing 400 mM L-arginine in PBS at 4°C and subsequent dialysis against PBS.

Cell-viability assay

The *in vitro* cell-killing activity of scFv(FRP5)-ETA was measured basically as described (Wels *et al.*, 1992). Human tumor cells or rat NV2Cd-erbB2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in normal growth medium. Various concentrations of scFv(FRP5)-ETA protein were added to triplicate samples and the cells were incubated for 40 hr (human tumor cells) or 96 hr (NV2Cd-erbB2). We added 10 µl of 10 mg/ml MTT (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide) (Sigma, Deisenhofen, Germany) in PBS to each well and incubated the cells for another 3 hr. Cells were lysed for 3 hr by the addition of 90 µl of 20% SDS in 50% dimethyl formamide, pH 4.7. The absorption at 590 nm was determined in a microplate reader (Dynatech, Denkendorf, Germany) as a measure of the number of viable cells in comparison with cells grown in the absence of recombinant protein.

In vivo *anti-tumor activity of scFv(FRP5)-ETA in immunocompetent rats*

Female BDIX rats (weight approximately 350 g) were purchased from Charles River (Sulzfeld, Germany). NV2Cd or NV2Cd-erbB2 cells (1×10^7) were re-suspended in 100 µl of PBS and injected s.c. into the flanks of the animals (3 rats/group). After 7 days, when the tumors were palpable, the animals were treated either by intratumoral or by intravenous injection of purified scFv(FRP5)-ETA or control proteins for 10 consecutive days. For local treatment, 50 µl each of the antibody-toxins were injected directly into 2 opposite sides of the tumor at total daily doses of 12.5 and 25 ins into the tail vein at daily doses of 125 and 250 µg/kg, corresponding to approximately 44 and 88 µg per injection for a 350-g rat. Tumor diameters were measured once or twice weekly with a calliper, and tumor volumes were calculated according to the formula: length × $(\text{width})^2 \times 0.5$ (Arteaga *et al.*, 1993).

Analysis of rat sera and preparation of cyst fluid

Blood samples were taken from the tail vein of rats. After clotting at 4°C for 16 hr and centrifugation at 8,000*g* for 20 min at 4°C serum was collected and stored at −20°C. After 15 µg of scFv(FRP5)-ETA or the irrelevant control protein scFv(gE10)-ETA were separated by SDS-PAGE on a 10% gel, they were blotted onto PVDF membranes (Millipore, Eschborn, Germany). The filters were probed with sera diluted 1:100 from tumor-bearing rats treated either with scFv(FRP5)-ETA or with PBS. As a positive control, a rabbit anti-ETA serum was used at 1:4,000 dilution. Binding of antibodies was detected by incubation of the filters with species-specific horseradish-peroxidase-labeled secondary antibodies and chemiluminescent detection using the ECL system (Amersham, Braunschweig, Germany). Cyst fluid was collected from established tumors by aspiration, centrifuged at 10,000*g* for 30 min and processed as described (Altenschmidt *et al.*, 1997).

RESULTS

Expression and purification of scFv(FRP5)-ETA

The 67-kDa antibody toxin scFv(FRP5)-ETA consists of an erbB2-specific scFv domain genetically fused to truncated *Pseudomonas* exotoxin A (Wels *et al.*, 1992). The ETA fragment represents the toxin's translocation domain II and the enzymatic domain III (amino acids 252 to 613) which, upon binding to target cells, facilitate uptake into the cytosol and inhibition of cellular protein synthesis (Hwang *et al.*, 1987; Ogata *et al.*, 1992). At the N-terminus, the recombinant protein carries a synthetic FLAG epitope followed by a cluster of 6 histidine residues for purification via Ni^{2+} affinity chromatography. In an earlier study, the scFv(FRP5)-ETA protein was expressed in *E. coli* and directed to the periplasmic space via the ompA signal peptide included in the expression plasmid pSW202-5 (Wels *et al.*, 1995). In order to allow the accumulation of large amounts of the recombinant protein as inclusion bodies in the cytoplasm of *E. coli* here, the ompA signal sequence was removed from the plasmid pSW202-5, resulting in the construct pSW220-5. This plasmid facilitates expression of a scFv(FRP5)-ETA molecule, which is identical to the previously used protein except for the lack of the secretion signal.

The modified expression plasmid was transformed into *E. coli* strain HB101, and expression of the recombinant protein was induced by the addition of 1% lactose. Protein expression peaked 16 to 20 hr after induction, in contrast to 3 hr when the synthetic IPTG was used for induction (data not shown). Cells were harvested, inclusion bodies were isolated and solubilized in a buffer containing 8 M urea, 50 mM Tris, pH 9.0, and 250 mM NaCl. Decreasing the pH of the buffer resulted in a lower yield of solubilized recombinant protein, whereas a lower NaCl concentration negatively influenced the binding of denatured scFv(FRP5)-ETA to Ni^{2+}-saturated chelating sepharose in the subsequent purification step (data not shown). ScFv(FRP5)-ETA was purified from solubilized inclusion bodies via a single round of Ni^{2+} affinity chromatography and re-natured by rapid dilution and subsequent dialysis as, described in ''Material and Methods''. The yield of purified protein was typically 30 mg/l of original bacterial culture, with a purity of more than 90% as determined by SDS-PAGE analysis (data not shown).

Cytotoxic activity and specificity of scFv(FRP5)-ETA

In order to determine the cytotoxic activity and specificity of scFv(FRP5)-ETA purified from inclusion bodies, *in vitro* cell-killing experiments were performed. SKBR3 and MDA-MB468 human breast-carcinoma cells and A431 human epidermoid-tumor

viable cells in comparison with cells grown in the absence of toxin was determined. The results are summarized in Table I. ScFv(FRP5)-ETA purified from inclusion bodies was cytotoxic for SKBR3 cells, which highly over-express erbB2 on their surface (IC_{50} of 50 ng/ml). The antibody toxin did not affect the growth of MDA-MB468 cells, which express high levels of the EGF receptor but only very low levels of erbB2, indicating that it is highly specific for erbB2-expressing cells. ScFv(FRP5)-ETA was also active on A431 cells, which express approximately 5×10^4 erbB2 molecules per cell. We have shown that A431 cells, despite moderate erbB2 levels, are highly sensitive to scFv(FRP5)-ETA, probably due to increased receptor turnover caused by heterodimerization of erbB2 with EGF receptor, which is highly over-expressed in A431 cells and activated via autocrine stimulation by TGF-α (Wels *et al.*, 1995; Schmidt *et al.*, 1996). As shown in Table I, the *in vitro* cell-killing activity of scFv(FRP5)-ETA expressed from a construct lacking the ompA signal sequence and purified from inclusion bodies was very similar to that of the recombinant protein used in earlier studies. This indicates that the properties of the antibody toxin were not altered by the different way of expression and purification.

In vitro *cell-killing activity of scFv(FRP5)-ETA*

Implantation of NV2Cd rat schwannoma cells in syngeneic BDIX rats initially results in a strong tumor-specific cytotoxic-T-cell response (Altenschmidt *et al.*, 1997). However, the tumor cells eventually escape elimination by the host immune system, through the secretion of immunosuppressive factors such as isoforms of TGF-β. In this respect our model resembles a possible clinical situation, since in certain cases anti-tumor immune responses observed in cancer patients are not sufficient to accomplish tumor rejection. In contrast, a tumor-specific antibody toxin should not be affected in its activity by immunosuppressive agents. NV2Cd-erbB2 cells have been generated by Nikitin *et al.* (1996) via stable transfection of NV2Cd cells with a human *erb*B2 cDNA construct. The resulting cells display *in vitro* and *in vivo* growth characteristics indistinguishable from the parental cell line (data not shown).

The expression of human erbB2 by NV2Cd-ErbB2 cells was confirmed by immunoblot analysis with a monoclonal antibody (MAb) specific for human erbB2 (Fig. 1*a*). The binding of the scFv(FRP5)-ETA immunotoxin to the surface of NV2Cd-erbB2 cells was verified by FACS analysis (data not shown). The *in vitro* cytotoxic activity of scFv(FRP5)-ETA on NV2Cd-erbB2 schwannoma cells was analyzed in cell-killing experiments. The cells were incubated for 96 hr with the antibody toxin at concentrations ranging from 1 ng/ml to 10 μg/ml, and the relative number of viable cells was determined. The results are shown in Figure 1*b*. In contrast to the erbB2-over-expressing tumor cell lines shown in Table I, NV2Cd-erbB2 cells were only moderately sensitive to scFv(FRP5)-ETA *in vitro*, which might reflect their lower level of erbB2 expression of approximately 1.5×10^5 receptors/cell. However, parental erbB2-negative NV2Cd cells remained unaffected by the immunotoxin, indicating that the growth inhibitory effect was specific (Fig. 1*b*). As shown for human erbB2-expressing tumor cells (Harwerth *et al.*, 1992) the parental erbB2-specific antibody FRP5 in the absence of the cytotoxic ETA effector domain had no effect on the growth of NV2Cd-erbB2 cells (data not shown).

In vivo *anti-tumoral activity of scFv(FRP5)-ETA*

BDIX rats carrying established s.c. NV2Cd-erbB2 tumors (3 animals/group) were treated with the erbB2-specific scFv(FRP5)-ETA toxin. NV2Cd-ErbB2 schwannoma cells (1×10^7) were injected s.c. into the flanks of syngenic immunocompetent rats. After 7 days, when the tumors were palpable, the animals were treated with scFv(FRP5)-ETA for 10 days and the effects of local and systemic treatment on tumor growth were investigated. The results are shown in Figure 2. Intratumoral application of 25 μg/day resulted in significant growth inhibition of NV2Cd-erbB2 tumors in comparison with PBS-treated controls, whereas treatment with a lower daily dose of scFv(FRP5)-ETA (12.5 μg/day) was not

TABLE I – *IN VITRO* CELL-KILLING ACTIVITY OF scFv(FRP5)-ETA PURIFIED FROM DIFFERENT SUB-FRACTIONS

Cell line	Number of erbB2 receptors[1]	IC_{50} (ng/ml)[2]	
		Total lysates[3]	Inclusion bodies
A431	5×10^4	33	18
MDA-MB468	$<5 \times 10^3$	>1000	>1000
SKBR3	$1–2 \times 10^6$	34	50

[1]ErbB2-receptor numbers were reported by Jannot *et al.* (1996).–[2]IC_{50} values were determined in a cell viability assay, as described in "Material and Methods".–[3]The cell-killing activity of scFv(FRP5)-ETA purified from total cell lysates were determined earlier (Wels *et al.*, 1995).

doses of 125 or 250 μg/kg for 10 consecutive days (Fig. 2*b*). Systemic treatment with 250 μg/kg/day inhibited NV2Cd-erbB2 tumor growth, but was less effective than local treatment with 25 μg/day. Treatment with 125 μg scFv(FRP5)-ETA/kg/day had no effect on *in vivo* tumor growth. Upon termination of the treatment, the tumors regrew in all animals. The antibody toxin was well tolerated by the animals, and no weight loss or other signs of systemic toxicity were observed (data not shown).

Specificity of scFv(FRP5)-ETA in vivo

In order to confirm that the anti-tumoral effects of scFv(FRP5)-ETA were mediated via specific binding of the toxin to erbB2, a similar experiment was performed with animals carrying tumors of the parental NV2Cd cells, which do not express human erbB2. Established s.c. NV2Cd tumors in BDIX rats were treated by intratumoral injection of 25 μg/day of scFv(FRP5)-ETA for 10 consecutive days. As controls, animals carrying NV2Cd-erbB2 tumors were treated either with the same dose of scFv(FRP5)-ETA or with PBS (3 animals/group). The results are shown in Figure 3*a*. As observed before, treatment with 25 μg/day of the antibody toxin inhibited the growth of NV2Cd-erbB2 tumors. In contrast, the growth of erbB2-negative NV2Cd tumors was not affected by scFv-(FRP5)-ETA, indicating that the antibody toxin is strictly dependent on erbB2 on the tumor cell surface to elicit a therapeutic effect.

To further exclude the possibility that unspecific toxic effects contribute to the observed inhibition of tumor growth, established NV2Cd-erbB2 tumors were treated as described above by intratumoral injection of 25 μg/day of the antibody toxin scFv(225)-ETA for 10 consecutive days. ScFv(225)-ETA is very similar in its structure to scFv(FRP5)-ETA, but is selectively cytotoxic for human tumor cells over-expressing the EGF receptor (Wels *et al.*, 1995). As shown in Figure 3*b*, at an identical dose the EGF-receptor-specific antibody toxin, in contrast to scFv(FRP5)-ETA, had no effect on the *in vivo* growth of NV2Cd-erbB2 tumors. These results clearly show that the growth inhibitory effects of scFv(FRP5)-ETA are highly specific.

Effect of treatment schedule on the anti-tumoral activity of scFv(FRP5)-ETA

To analyze the dependency of scFv(FRP5)-ETA anti-tumoral activity on the period of tumor establishment before treatment, BDIX rats carrying s.c. NV2Cd-erbB2 tumors were treated by intratumoral injection of 25 μg/day of scFv(FRP5)-ETA either from day 7 to day 16 or from day 13 to day 22 after implantation of the tumor cells. Control animals were treated with PBS from day 13 to day 22. The results are shown in Figure 4. Tumor growth was suppressed by scFv(FRP5)-ETA during the treatment period when the treatment was begun on day 7. In contrast, when the tumors were larger at the onset of treatment on day 13, no significant inhibition of tumor growth at the same dose of scFv(FRP5)-ETA was observed in comparison with PBS-treated controls.

We have shown that NV2Cd tumors develop necrotic areas inside the tumor approximately 15 days after s.c. implantation of cells and form fluid-filled cysts (Altenschmidt *et al.*, 1997). Proteolytic activity in the cyst fluid could be responsible for rapid

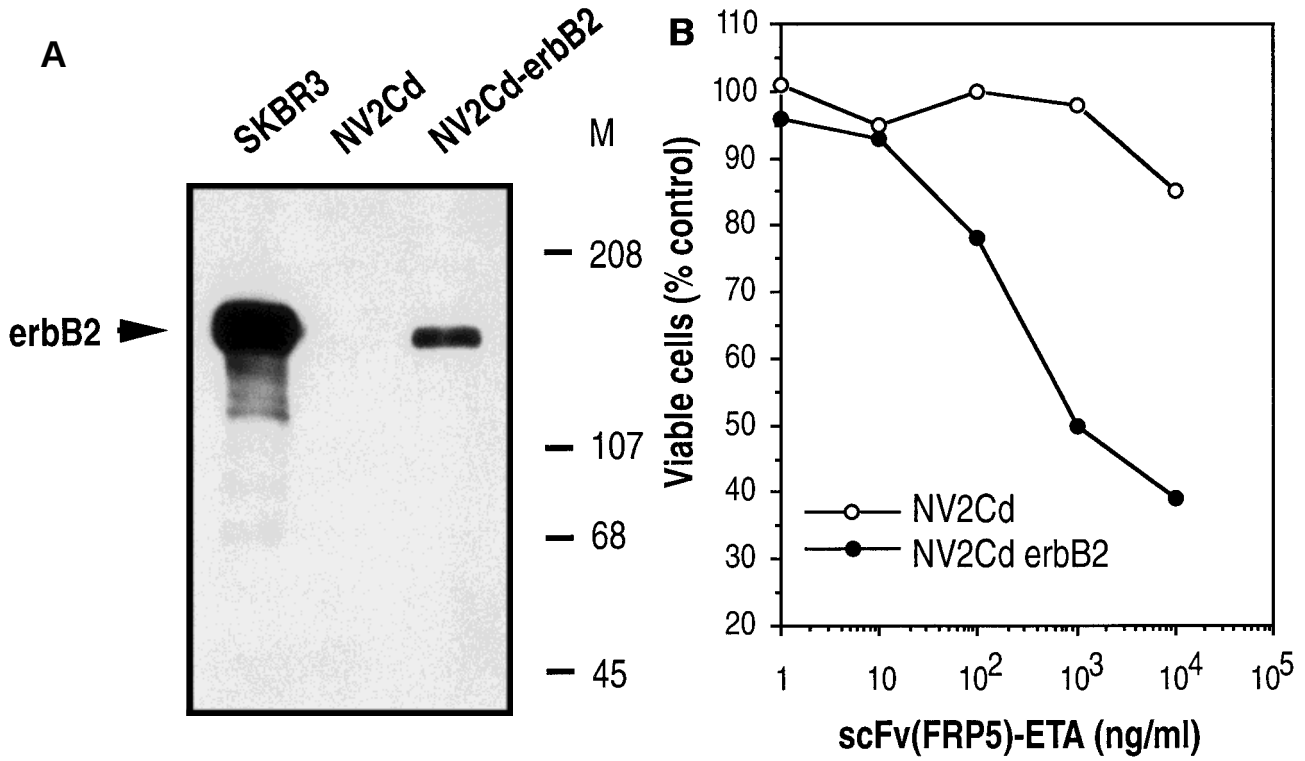

FIGURE 1 – *(a)* Immunoblot analysis of cell lysates from NV2Cd transfectants. Equal amounts of cell lysates from NV2Cd-erbB2 rat schwannoma cells stably transfected with human *erb*B2 cDNA, parental NV2Cd cells, and erbB2-over-expressing SKBR3 human mammary-carcinoma cells were separated by electrophoresis on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes as indicated. Immunodetection was performed with a MAb specific for human erbB2, followed by incubation with an anti-mouse horseradish-peroxidase-labeled antibody and chemiluminescent detection. The position of the erbB2 protein is indicated. *(b)* Inhibition of the *in vitro* growth of NV2Cd-erbB2 schwannoma cells. NV2Cd-erbB2 cells and erbB2-negative parental NV2Cd cells were incubated for 96 hr with the indicated concentrations of the erbB2-specific scFv(FRP5)-ETA, and the number of viable cells in comparison with PBS-treated cells was determined by an enzymatic assay, as described in "Material and Methods".

bated *in vitro* for different time intervals with cyst fluid removed from 23-day-old tumors at a ratio of 1 µl of cyst fluid per µg of protein. The integrity of the fusion protein was subsequently analyzed by SDS-PAGE and immunoblotting with a specific antibody. As shown in Figure 5, the immunotoxin was partially degraded after 2 hr of incubation with cyst fluid at 37°C (lane B) and completely degraded after 4 hr of incubation (lane C). These results show that proteolytic activity is present in the necrotic areas of NV2Cd-erbB2 tumors, and rapidly destroys the scFv(FRP5)-ETA immunotoxin. This explains the failure of the antibody toxin to inhibit the growth of tumors at time points when cysts have already formed.

Specific antibody response in scFv(FRP5)-ETA-treated animals

After termination of the *in vivo* experiments, serum was obtained from the tumor-bearing rats, in order to investigate the generation of immunotoxin-specific antibodies. Purified scFv(FRP5)-ETA was separated by SDS-PAGE, blotted onto PVDF membranes, and incubated with rat sera. The results are shown in Figure 6*a*. Both, sera from rats treated systemically with different doses of scFv-(FRP5)-ETA (lanes 1 and 2) and sera from animals treated by intratumoral injection (lanes 3 and 4) contained anti-immunotoxin antibodies with higher titers in the sera of systemically treated rats. As expected, no anti-scFv(FRP5)-ETA antibodies developed in PBS-treated animals (lane 5). The antibodies were directed mainly against the toxin domain, since the sera reacted equally with an irrelevant scFv(9E10)-ETA control protein containing a different antibody domain specific for a myc epitope (data not shown) (Fig. 6*b*, lanes 8–11). Neither PBS-treated animals nor animals treated with the immunotoxin developed antibodies directed against the human erbB2 molecule (data not shown).

carrying s.c. NV2Cd-erbB2 tumors were treated for 10 days as described above by intratumoral injection of 25 µg/day of scFv-(FRP5)-ETA pre-treated with the serum obtained from toxin-treated animals, at a concentration of 20 µl serum/mg recombinant antibody toxin. As shown in Figure 6*c*, no significant reduction in the anti-tumoral activity of scFv(FRP5)-ETA was observed upon pre-treatment with the rat serum as compared with the results of treatment with the native antibody toxin. This is consistent with the results of *in vitro* cell-killing experiments with erbB2-over-expressing human tumor cells and scFv(FRP5)-ETA in the presence of rat sera. In these experiments, no difference in the effects of sera from toxin-treated or PBS-treated animals on the cytotoxic activity of the immunotoxin was observed (data not shown), indicating that upon treatment with the recombinant toxin a humoral anti-toxin response develops. However, this response did not result in neutralization of the anti-tumoral activity of the fusion protein.

DISCUSSION

Members of the erbB family of receptor tyrosine kinases have been shown to play an important role in tumor development and progression. In particular, over-expression of erbB2 and the EGF receptor has been correlated with poor clinical outcome in sub-sets of human malignancies (Hynes and Stern, 1994; Gullick, 1991). Due to the differential expression of such receptors, high on the tumor cell surface and low on most normal tissues, therapeutic strategies specifically targeted to erbB2 and the EGF receptor hold promise to improve the clinical situation. MAbs specific for the extracellular domains of such receptors have been shown to

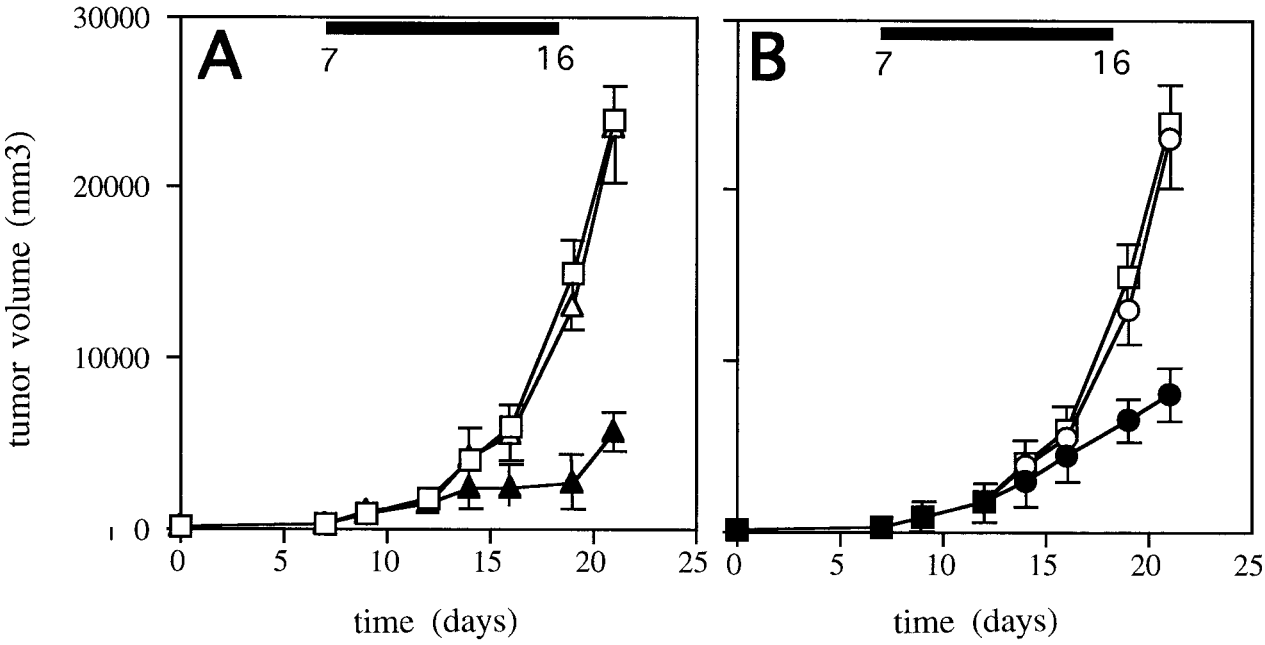

FIGURE 2 – Effect of scFv(FRP5)-ETA on the *in vivo* growth of NV2Cd-erbB2 schwannoma cells. Tumor cells (1×10^7) were injected s.c. in syngeneic BDIX rats on day 0. From day 7 to day 16 the animals received daily injections of scFv(FRP5)-ETA directly into the tumor *(a)* of 25 µg/day (filled triangles) or of 12.5 µg/day (open triangles), or systemically into the tail vein *(b)* of 250 µg/kg/day (closed circles) or of 125 µg/kg/day (open circles). Control animals were treated with PBS (open squares). Tumor size was measured at the indicated time points and tumor volumes were calculated. The mean values for each group are shown and the standard deviation is represented by error bars.

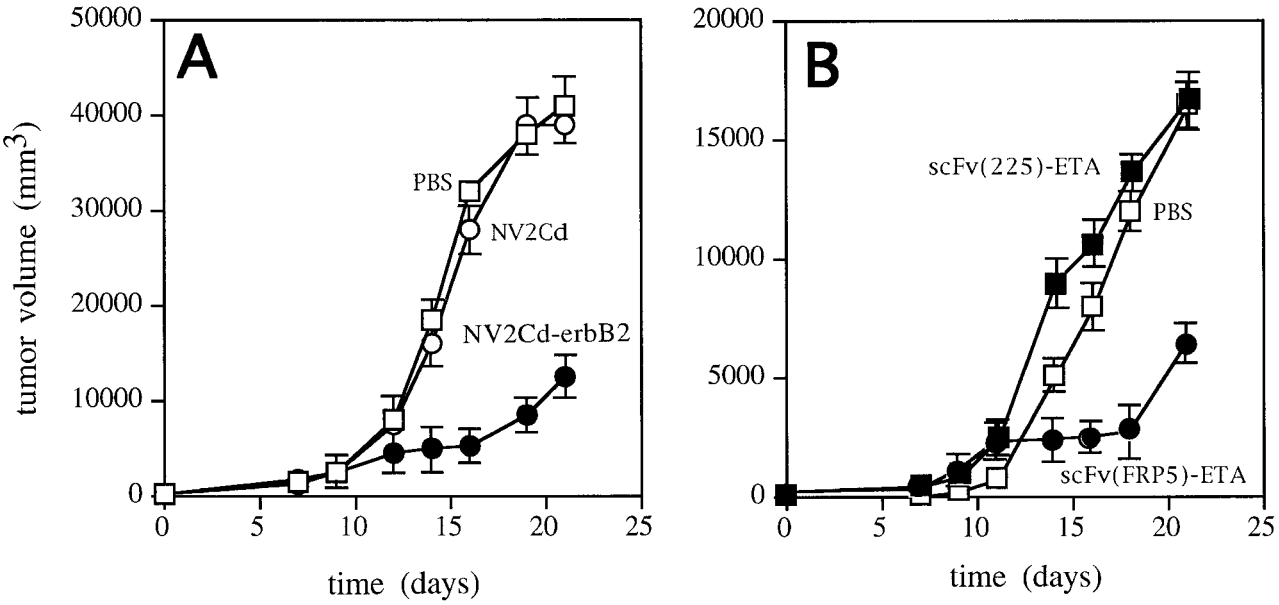

FIGURE 3 – Specificity of scFv(FRP5)-ETA *in vivo*. *(a)* NV2Cd-erbB2 (closed circles) or erbB2-negative parental NV2Cd schwannoma cells (open circles) (1×10^7 in each case) were injected s.c. in syngeneic BDIX rats on day 0. From day 7 to day 16 the animals received daily intratumoral injections of 25 µg/day of scFv(FRP5)-ETA. Control animals were treated with PBS (open squares). *(b)* NV2Cd-erbB2 tumor cells (1×10^7) were injected s.c. in syngeneic BDIX rats on day 0. From day 7 to day 16 the animals received daily intratumoral injections of 25 µg/day of the erbB2-specific scFv(FRP5)-ETA (closed circles) or the EGF-receptor-specific control protein scFv(225)-ETA (closed squares). Control animals were treated with PBS (open squares). Tumor size was measured at the indicated time points and tumor volumes were calculated. The mean values for each group are shown and the standard deviation is represented by error bars.

sohn, 1994; Baselga *et al.*, 1996). The growth-inhibitory potential of such antibodies can be potentiated by connecting them to cytotoxic effector functions such as bacterial toxins. We have described the potent anti-tumoral activity of recombinant single-

Here we have studied the anti-tumoral activity of the erbB2-specific scFv-*Pseudomonas* exotoxin-A fusion protein scFv(FRP5)-ETA in immunocompetent rats. The *in vivo* effects of molecules such as immunotoxins containing species-specific binding domains

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