Recombinant Immunotoxins Directed against the c-erb-2/HER2/neu Oncogene Product: In Vitro Cytotoxicity, Pharmacokinetics, and in Vivo Efficacy Studies in Xenograft Models

Michael G. Rosenblum, Laura K. Shawver, John W. Marks, Jody Brink, Lawrence Cheung, and Beatrice Langton-Webster

Section of Immunopharmacology and Targeted Therapy, Department of Bioimmunotherapy, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [M. G. R., J. W. M., L. C.], and Department of Oncology, Berlex Biosciences, Richmond, California 94804-0099 [J. B.]

ABSTRACT

TAB-250 and BACH-250 are murine and human chimeric antibodies directed at the extracellular domain of the gp185\textsuperscript{-}erb-2 (HER2/neu) growth factor receptor overexpressed in a variety of tumor types, including ovarian and breast carcinoma. The ribosome-inhibiting plant toxin gelomin (rGel) was chemically coupled to both antibodies, and the resulting immunotoxins were purified and tested in vitro against human tumor cells expressing various levels of HER-2/neu and in vivo against human tumor xenograft models. The binding of both BACH-250 and BACH-250/rGel conjugate to target cells was essentially equivalent. Against SKOV-3 cells, the IC\textsubscript{50} of BACH-250/rGel was 97 pm (17 ng/ml), whereas BACH-250 and rGel alone showed no cytotoxic effects. There was a clear correlation between expression levels of HER-2/neu and cytotoxicity. Tissue distribution studies showed that the antibody and immunotoxin both concentrate 2–10-fold higher in tumors than in normal tissues, with optimal tumor uptake occurring 48–96 h after administration. Plasma clearance curves for BACH-250 and BACH-250/rGel showed terminal-phase half-lives of 26 and 72 h, respectively. In athymic mice bearing s.c. or i.p. SKOV-3 tumors, immunotoxin treatment slowed tumor growth by 99 and 94% at days 35 and 49 after implantation, respectively, and lengthened the median survival by 40% (from 30 to 50 days) in mice bearing lethal i.p. tumors. We conclude that clinical development of BACH-250/rGel may be warranted in patients with HER2/neu-expressing malignancies.

INTRODUCTION

A variety of studies have recently described numerous molecular markers important in predicting eventual responses to therapy and the natural course of breast cancer and ovarian carcinoma (1–5). One marker, c-erb-2/Her-2/neu, has been found to be associated with a poor prognosis for a variety of tumor types. This proto-oncogene was found to encode a M\textsubscript{r} 185,000 transmembrane glycoprotein tyrosine kinase (gp 185) and to show extensive homology with epidermal growth factor receptor (6–11). In addition, transfection studies have shown that HER-2 overexpression may play a direct role in the transformation of the phenotype from a normal to neoplastic one (12, 13). Amplification of the gene or overexpression of gp 185 has been well described in numerous human cancers, including mammary and ovarian carcinomas, gastric tumors, and adenocarcinomas of the colon and salivary gland (14–16). In particular, Slamon et al. (17, 18) found HER-2/neu overexpressed in −30% of 189 primary breast carcinomas studied. More importantly, they noted that the overexpression of HER-2/neu correlated with a poor prognosis. Follow-up studies have also shown that HER-2 appears to be associated with a shortened disease-free survival (19, 20). Thus, the clinical observations regarding the importance of HER-2/neu as a negative prognostic indicator have been repeatedly confirmed by molecular studies, demonstrating the central role of this oncogene in promoting the growth of transformed cells and in increasing their metastatic potential. Because of the key role of HER-2/neu in transforming the phenotype, its surface expression on breast tumors, and its relatively restricted expression in normal adult tissues, numerous investigators have suggested that this protein may be a useful therapeutic target in a subset of breast and ovarian carcinomas.

Unlike the heterogeneous intratumoral expression of other tumor-associated antigens, the intratumoral expression of HER2/neu appears to be uniform (21). In addition, tumor expression of HER2/neu in primary and metastatic lesions appears constant over time within the same patient. Therefore, escape and repopulation of antigen-negative tumor cells after treatment with anti-HER-2-directed therapeutic agents appear reduced, and this suggests that this cell-surface protein may be extremely useful for targeted therapies. Numerous monoclonal antibodies targeting the gp185 cell-surface domain have recently been developed (22, 23), providing a range of opportunities for targeted immunotherapeutic drug development. One such agent, the human chimeric antibody 4D5, which recognizes gp185, is now in Phase III clinical trials (24) and has shown a 20–30% response rate in breast cancer patients in Phase I/I trials.

In the present study, we used molecular techniques to...
design unique immunotargeted proteins recognizing gp 185 and to examine the efficacy of these constructs using a variety of preclinical models. Because these constructs contain a recombinant antibody with significant murine sequences that have been replaced with human sequences, we anticipate that these agents will have a considerably less antigenic effect than other previously described immunotoxins, thereby allowing for a prolonged therapeutic clinical window.

MATERIALS AND METHODS

Materials

The reagents 4-succinimidyl-oxycarbonyl-methyl-α-[2-pyridyldithio]toluene, 3–4,5-dihydro-6-(4-(3,4-dimethoxybenzoyl)-1-piperazinyl-2(ih)-quinolinone, and dimethylformamide were obtained from Sigma Chemical Corp. Mammalian cell culture medium was purchased from Life Technologies, Inc. (Gaithersburg, MD), and fetal bovine serum was purchased from Hyclone Labs, Inc. (Logan, UT). TAB-250, a murine IgG2b monoclonal antibody that recognizes the c-erb-2 surface domain (25) and BACH-250, a recombinant mouse:human chimeric antibody developed by fusing the murine hypervariable light and heavy chain domains to a human immunoglobulin framework, were supplied as purified reagents by Berlex Biosciences, Inc. (Alameda, CA; Ref. 26). The affinities and reactivities of the two antibodies with the c-erb-2 cell-surface oncoprotein are similar. The recombinant gelolin toxin (rGel) was produced as a fermentation product in Escherichia coli and purified as described previously (27). SPDP reagent and 2-iminothiolane HCl were purchased from Pierce Chemical Co. (Rockford, IL). Sepharose S-300 gel permeation resin and blue Sepharose CL-6B were purchased from Pharmacia (Piscataway, NJ). A rabbit reticulocyte translation kit (Amersham, Arlington Heights, IL) was used to estimate of the cell-free protein synthesis inhibitory activity of the toxin (27). PBS was also purchased from Life Technologies, Inc.

Methods

Immunotoxins with TAB-250 or BACH-250

The details for the generation of antibody conjugates and rGel have been published elsewhere (28, 29). Briefly, a stock solution of SPDP in dry dimethylformamide was added to a solution of either TAB-250 or BACH-250 to a final concentration of 5-fold molar excess. Excess unreacted SPDP was removed by Sephadex G-25 chromatography. SPDP-derivitized antibody fractions were pooled and kept at 4°C. One mg of purified rGel (2 mg/ml in PBS) was added to TEA/HCl buffer to a final concentration of 60 mM TEA/HCl, adjusted to pH 8.0, and then EDTA was added to a concentration of 1 mM. A 2-iminothiolane stock solution was added to a final concentration of 1 mM, and the sample was incubated for 90 min at 4°C under nitrogen. Excess 2-iminothiolane was removed by gel filtration. SPDP-modified antibody was mixed with an equal weight of 2-iminothiolane-modified rGel, which corresponded with a 5-fold molar excess of gelolin as compared with antibody. The pH of the mixture was adjusted to 7.0 by adding 0.5 mM TEA/HCl buffer (pH 8.0), and the mixture was incubated for 20 h at 4°C under nitrogen. Iodoacetamide (0.1 mM in H2O) was added to a final concentration of 2 mM to block any remaining free sulfhydryl groups, and incubation was continued for an additional hour at 25°C.

To remove low molecular weight products and nonconjugated rGel, the reaction mixture was applied to a Sepharcl S-300 column (1.6 × 31 cm) previously equilibrated with PBS. Fractions were collected, and the protein content was measured. The high molecular weight peak fractions were applied to an affinity chromatography column of blue Sepharose CL-6B (1 × 24 cm) preequilibrated with 10 mM PBS (pH 7.2) containing 0.1 mM sodium chloride. The column then was washed with 50 ml of buffer to completely elute the nonconjugated antibody. This was done with a linear salt gradient of 0.1–2 mM sodium chloride in 10 mM PBS (pH 7.2). The protein content of the eluted fractions was determined using the dye-binding assay described previously (30), and the samples were analyzed using nonreducing SDS-PAGE.

Cell Culture Methods

Cell lines were maintained in culture in complete medium at 37°C in a 5% CO2-humidified air incubator. Minimal essential medium was used and supplemented with 10% heat-inactivated fetal bovine serum, plus 100 μM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, vitamins, and antibiotics. Cultured cells were routinely screened for Mycoplasma and found free of this bacterium.

Log-Phase Cytotoxicity Assays

For the assays with immunotoxins, cultures were washed, and cells were detached using Versene (edetate sodium), after which the cells were resuspended in complete medium at a density of 5 × 104 cells/ml. Aliquots of suspended cells were

---

5 The abbreviations used are: SPDP, succinimidyl-3-(2-pyridyldithio)propionate; TEA/HCl, triethanolamine hydrochloride; TNF, tumor necrosis factor.
then dispensed into 96-well microtiter plates, and the cells were allowed to adhere for 24 h. The medium was then replaced with medium containing different concentrations of immunotoxin or rGel, and the cells were incubated for an additional 72 h. The relative cell proliferation was analyzed using the 3–4,5-dihydro-6-(4-(3,4-dimethoxybenzoyl)-1-piperazinyl-2(ih)-quinolinone staining described previously (31). The values given are the means of duplicate experiments performed in quadruplicate.

**Iodination of Antibodies and Immunotoxins.** Briefly, to iodinate the antibodies and immunotoxins, 1 mCi of Na$^{125}$I, 17.4 mCi/mg (DuPont NEN Research, Boston, MA) was added to 20 µl of 0.1 M Na$^{2}$HPO$_4$, pH 7.4. The mixture then was added to a reaction vial coated with 10 µl of iodogen (previously dried in chloroform). The reaction was allowed to proceed for 15 min at room temperature. Unreacted radioiodine was removed by chromatography on a Sephadex G-25 (PD10) column. More than 90% of the radiolabel was incorporated into the protein complex, as measured by precipitation with trichloroacetic acid.

**Internalization of Immunotoxins.** The internalization of 125$I$-TAB-250 or 125$I$-TAB-250/rGel was assessed by determining the amount of radioactivity in acid-sensitive and -insensitive compartments. Cells were harvested and resuspended in buffer containing radiolabeled antibody alone or containing excess unlabeled antibody to determine the extent of nonspecific binding. After the cell-surface binding of the radiolabeled antibody reached equilibrium, the cells were centrifuged, and the pellet was washed to remove unbound antibody. The cells were warmed to 37°C to initiate internalization of the radiolabeled antibody. At the times indicated, aliquots were removed, and the cells were collected by centrifugation. The supernatants that contained dissociated or recycled antibody were collected. The pellets were resuspended twice in an acid wash, and the supernatants containing the surface-bound antibody were combined and counted. The tips of the centrifuge tubes containing the remaining cell-associated radioactivity were then clipped and counted.

**Displacement Studies**

To examine the displacement of 125$I$-labeled BACH 250 and 125$I$-BACH 250/rGel binding, SKOV-3 cells were grown to 80–90% confluence and harvested. A total of 3.6 × 10$^5$ cells were incubated on ice for 4 h in the presence of 5–10 ng/ml of 123$I$-labeled BACH-250 or BACH-250/rGel alone, or in combination with increasing amounts of unlabeled mouse IgG, BACH-250 or BACH-250/rGel for 4 h. Cells then were washed, and cell-associated radioactivity was determined using a gamma counter. The percentage of cells bound was determined by dividing the cpm measured by total cpm and then multiplying the quotient by 100.

**Animal Model Studies**

**Pharmacokinetics.** BALB/c mice, 4–6 weeks of age, were injected with 0.3 µCi (5 µg) of either labeled monoclonal antibody or immunoconjugate. Three mice each were sacrificed by cervical dislocation at 15, 30, 45, 60, 75, 90, 105, 120, and 240 min and 24 h after injection. Blood samples were removed (chest cavity) and weighed, and radioactivity was determined with a gamma counter. The blood samples then were centrifuged, and supernatant was decanted and counted to determine the amount of the plasma-associated radiolabel. The amounts of radioactivity were analyzed by a least-squares nonlinear regression program (RSTRIP; MicroMath, Inc.) to determine the pharmacokinetic parameters.

**Tissue Distribution**

BALB/c athymic mice received s.c. implants of SKOV-3 (positive for c-erbB-2 expression) and MDA-MB-468 (negative for c-erbB-2 expression) tumor cells on the left and right hind flanks, respectively. Tumors were allowed to grow to 0.1–0.4 g, at which time 10 animals each were injected i.p. with ~10 µg of either 125$I$-TAB-250 or 125$I$-TAB-250-gelonin (2–8 µCi/µg). Both of the iodinated proteins have been shown to compete comparably for the binding of unlabeled TAB-250 on SKOV-3 cells. Two animals from each group were then sacrificed at 6, 18, 24, 30, and 48 h; tumor and normal tissues were removed;
the samples were weighed, and radioactivity was counted. The amount of radiolabeled antibody distributed to the tumor or various organs was expressed as the percentage of the injected dose per gram of tissue and finally expressed as a ratio to the concurrent blood levels.

To examine the effects of TAB-250 or TAB-250/rGel on s.c. tumor growth, we implanted SKOV-3 tumor cells s.c. in BALB/c athymic mice (seven to eight mice/group). Test agents were administered i.p. three times per week for 3 weeks, starting on day 5 after implantation (tumor volumes, 0–30 mm³). Mice received PBS (control), TAB-250 (42 μg/dose) plus rGel (8 μg/dose), or the TAB-250-rGel conjugate (50 μg/dose). Tumors were measured twice per week with vernier calipers, and volumes (in cubic millimeters) were calculated as a product of the length × width × height.

To examine the effects of TAB-250 or TAB-250-rGel on i.p. tumor growth, we implanted i.p. SKOV-3 tumor cells in 60 BALB/c athymic mice. After implantation, the mice were randomly divided into five treatment groups of 12 mice each. Beginning on day 7, the mice in each group were given an i.p. injection of PBS, 107 μg of TAB-250 alone, 20.5 μg of rGel alone, 107 μg of TAB-250 plus 20.5 μg of rGel, or an equimolar amount of the TAB-250/rGel conjugate. A total of nine treatments were given, three times/week for 3 weeks, and survival time for each mouse was recorded.

RESULTS

Immunoconjugates with TAB-250 or BACH-250. rGel was covalently linked to either TAB-250 or BACH-250 using SPDP, a heterobifunctional cross-linking reagent. Analysis of the final purified sample using nonreducing SDS-PAGE showed that the final product contained a mixture of immunoconjugates containing one rGel molecule (major) and immunoconjugates containing two rGel molecules (minor). No demonstrable amounts of unconjugated antibody or free gelonin toxin were detected.

Cell Binding and Competition Studies. The binding of either unmodified TAB-250 or TAB-250/rGel to antigen-positive SKOV-3 cells as assessed by an ELISA showed that both reagents had a similar profile, suggesting that the binding determinants of the antibody are preserved after SPDP modification and toxin conjugation (Fig. 1). In competitive displacement studies using radiolabeled BACH-250 and BACH-250/rGel, the agents competed or cross-competed with either reagent, as shown in Fig. 2. Both radiolabeled BACH-250 and BACH-250/rGel bound to SKOV-3 target cells to the same extent, and the binding of neither agents could be displaced by nonspecific IgG. On the other hand, with the addition of unlabeled free antibody or immunotoxin, the binding of either radiolabel was competed identically. The affinity of the immunotoxin appeared to be identical to that of the original antibody, and the binding of the immunotoxin to target cells occurred solely through the interaction of the antibody component of the immunotoxin with the antigen on the target cells.

In Vitro Cytotoxicity Studies. Various concentrations of BACH-250/rGel were applied to log-phase SKOV-3 cells in culture in the presence of increasing concentrations of free antibody. As shown in Fig. 3, the immunotoxin was almost 100% cytotoxic to target cells at doses of up to 1 × 10⁻¹⁰ M. The IC₅₀ for the immunotoxin was 54.7 pM. In contrast, the IC₅₀ for free rGel under these conditions was 2 × 10⁻⁶ M, or a concentration almost 4 logs higher than that of the immunotoxin (data not shown). The coadministration of free antibody at doses of 0.01, 0.1, and 1.0 μg/ml increased the immunotoxin IC₅₀ to 63.5, 206, and 639 pM, respectively (see Fig. 2). Cell culture studies with the BACH-250/rGel immunotoxin showed that significant cytotoxicity occurred at concentrations of more than 0.01 μg/ml (Fig. 4). Maximal effects on cells were observed by day 6 of culture, and growth inhibition was observed for the 10 days of the observation period.

Previous studies performed in our lab with various cell lines expressing different levels of c-erb-B-2/HER-2/neu showed that antibodies TAB-250 and BACH-250 were not internalized in MDA-231 cells, which contain low levels of the c-erb-B-2 protein (∼0.16 × 10⁶ sites/cell). In contrast, SKBR-3 cells (≥2 × 10⁶ sites/cell) internalized the antibody most efficiently.
and the internalization of antibody into SKOV-3 cells ($\sim 1 \times 10^6$ sites/cell) and MDA-453 cells ($\sim 0.35 \times 10^6$ sites/cell) was intermediate (data not shown).

The cytotoxic effects of TAB-250/rGel were examined in six different cell lines that express various levels of the c-erbB-2 protein (Fig. 5). As predicted from the internalization studies, the cytotoxic activity of the conjugate was greatest against the SKBR-3 cell line with the highest number ($\sim 4 \times 10^6$ sites/cell) of cell-surface receptors. Intermediate toxicity was observed in SKOV-3 cells; further reduced cytotoxic effects were observed in MDA-MB-453 cells, and almost no cytotoxic effects were observed in MDA-231 or MCF-7 cells, which had the fewest number of sites/cell.

This experiment showed that in these cells, the cytotoxicity of the TAB-250/rGel immunoconjugate correlates well with the number of cell-surface receptors and that the internalization of cell-surface bound antibodies or immunotoxins may depend on the number of available c-erbB-2 receptors. Because rGel must be internalized to be cytotoxic, anti-HER-2 immunotoxins may be most effective in cells expressing very high levels of HER-2.

The pattern of the clearance of radiolabeled BACH-250 and BACH-250/rGel from the plasma of mice after i.v. administration is shown in Fig. 6. Although both agents were cleared from plasma in a similar fashion, pharmacokinetic analysis of the clearance curves (Table 1) showed that the BACH-250 antibody closely fit a one-compartment model for clearance, whereas the immunotoxin was cleared biphasically. In addition, the calculated terminal-phase half-lives of the antibody and the immunotoxin were 26.9 and 75.2 h, respectively. However, the initial concentrations of the two agents in plasma were similar, suggesting that there were no significant changes in the initial apparent volume of distribution. The overall clearance kinetics of the immunotoxin also were not influenced significantly by the toxin component but do appear to be related to the rate of clearance of the parent antibody.

The tissue distribution profiles of radiolabeled BACH-250 and BACH-250/rGel at 24, 48, 72, and 96 h after i.v. administration in athymic mice bearing SKOV-3 xenografts are shown in Figs. 7–10, respectively. In almost all of the tissues studied, the content of radiolabeled immunotoxin generally paralleled that of the parent antibody at all of the same points. However, at 24 and 48 h after administration, the content of immunotoxin in liver and spleen appeared to be higher than that of the parent antibody, indicating that the increased uptake may be due to the effects of toxin component of the immunotoxin construct. The content of both the antibody and the immunotoxin in normal tissues decreased significantly over time. In addition, as shown in Fig. 11, the content of the immunotoxin and the parent antibody were similar over time, reaching a plateau by $\sim 48$ h after administration and remaining for 96 h.

Results of treatment of BALB/c mice bearing well-established s.c. SKOV-3 tumors are shown in Fig. 12. Treatment with the TAB-250-rGel conjugate was shown to inhibit tumor growth by 99% at day 35 and up to 93% by day 55. In contrast, animals treated with the unconjugated combination of TAB-250 plus...
free rGel showed a maximal tumor inhibition of only 56% compared with the control group over days 35–55. Tumors regressed or did not develop in five of the seven animals in the immunotoxin (TAB-250/rGel) treatment group, whereas sizable tumors developed in all seven animals treated with either PBS or the unconjugated combination of TAB-250 plus rGel. This finding showed that tumor growth was inhibited significantly more in animals treated with the TAB-250/rGel conjugate than in control mice, (99 and 94% at day 49 after implantation, respectively), and also more so than it was in mice treated with equimolar amounts of unconjugated TAB-250 plus free rGel (4% at day 35 and 56% at day 49).

In a lethal model of SKOV-3 growing as an i.p. xenograft in BALB/c mice (Fig. 13), treatment with the TAB-250/rGel conjugate lengthened the survival of mice with these tumors by an average of 40% (12.4 days) as compared with survival in mice treated with PBS, TAB-250, or unconjugated TAB-250 plus rGel.

**DISCUSSION**

Although numerous antibodies have been described that react with the surface domain of the *HER-2/neu* proto-oncogene (32, 33), there have been surprisingly few reports of the development of immunotoxins from these reagents. However, both Tecce et al. (34) and Rodriguez et al. (35) described immunotoxins that target HER-2 and that contained the toxins saporin (34) and ricin A chain (RTA; 35). Both groups of investigators also noted that these constructs had impressive and selective *in vitro* antitumor effects on tumor cells expressing HER-2. The present study, which evaluated the behavior of immunotoxins containing the recombinant plant toxin gelonin, confirmed and extended these observations. In addition, we used a human chimeric anti-HER2/neu antibody in most of our studies in an effort to develop an immunotoxin with less antigenicity than...
immunotoxin. The greatest metastatic potential and the highest growth potential were observed in tumor cell lines that overexpress HER-2/neu, and therefore those with HER-2/neu should remain sensitive. This also suggests that those tumors affected by this construct, whereas overexpressing tumor cells expressing relatively low levels of HER-2/neu should not be susceptible. Internalization of the complex. Therefore, normal tissues expressed antibodies that of previously described constructs containing murine antibodies.

Of concern in the development of therapies that target HER-2/neu, however, is the expression of the target antigen on normal tissues. To determine whether this is a potential problem, Press et al. (36) evaluated a variety of normal adult and fetal tissues for protein HER-2/neu expression. Using immunohistochemistry and Northern blot analyses to identify the HER-2/neu protein and transcript, respectively, the cell-surface protein was identified on membranes of epithelial cells in the gastrointestinal, respiratory, reproductive, and urinary tracts, as well as in normal skin, breast, and placenta. This observation was confirmed by Northern hybridization. However, the amount of HER-2/neu transcript and protein was generally higher in fetal tissues than in the corresponding normal adult tissues. The levels of HER-2/neu produced in these normal tissues were also similar to the levels in nonamplified, nonoverexpressing breast cancers and breast cancer cell lines.

The present study clearly showed that tumor cells expressing HER-2/neu at levels less than 500,000 sites/cell were relatively insensitive to the cytotoxic effects of immunotoxin, whereas cells expressing at least 1 × 10^6 sites/cell were sensitive to the cytotoxic effects of this agent. This suggests that although the potential number of cell-surface targets are significant, the HER-2/neu antigen may be relatively poorly internalized upon antibody binding of the immunotoxin construct, or this may suggest that relatively few immunotoxin molecules are able to distribute to the correct intracellular compartment upon internalization of the complex. Therefore, normal tissues expressing relatively low levels of HER-2/neu should not be affected by this construct, whereas overexpressing tumor cells should remain sensitive. This also suggests that those tumors producing the highest levels of HER-2, and therefore those with the greatest metastatic potential and the highest growth potential, are the most sensitive to the antiproliferative effects of this immunotoxin.

Pharmacokinetic and tissue disposition analyses performed as part of our study showed that the BACH-250/rGel immunotoxins generally behave in vivo like the original antibody component. Immunotoxin was detected in tumor 24–48 h after injections and at a higher concentration than found in all efficacies examined. In normal tissues, the highest concentration of immunotoxin was found in the liver and spleen and did not appear to result in toxicity, because all mice appeared normal.

Studies in which rGel has been used in combination with other antibodies, such as the antimelanoma antibody ZME-018, have also shown that immunotoxins containing rGel localize in tumor xenografts and clear from the systemic circulation in a manner similar to that of the native antibody. These findings are in sharp contrast to the in vivo behavior of immunotoxins containing toxins such as RTA. Studies by Trown et al. (37) and Wawrzynczak et al. (38) showed that RTA-containing immunotoxins clear very rapidly from the systemic circulation as compared with the clearance kinetics of the parent antibodies. In addition, studies performed by Blakey et al. (39) have indicated that this rapid clearance may be mediated, at least in part, through the recognition of the carbohydrate clusters on the plant protein by cells of the reticuloendothelial system. In the present study, however, the rGel used for conjugation was derived from E. coli and therefore contained no carbohydrate groups. However, our initial studies with immunotoxins containing naturally derived, glycosylated gelonin also showed no significant uptake by elements of the reticuloendothelial system, suggesting that the glycosylation of gelonin may be unique compared with that of other plant-derived, ribosome-inhibiting toxins such as saporin and RTA.

For the in vivo studies, a tumor cell line (SKOV-3) was used that overexpresses HER-2/neu, but at levels that may approximate those found in patients in whom the oncoprotein is highly expressed. Under these circumstances, the immunotoxin was found to have impressive antitumor effects as compared with the tumor growth behavior seen in the control groups in both the s.c. model and the i.p. model. It is important to note,
however, that because of the limitations of materials, animals were given doses far below the maximal tolerated dose, and antitumor activity at higher doses was not explored. We expect that a greater antitumor effect of this agent will be observed when it is given at the maximal tolerated dose. In our animal model experiments, we also noted that the antibody alone had some growth inhibitory effect. As shown for other anti-HER-2 antibodies, this is likely due to antibody binding to the cell surface gp 185, in turn causing interference with the growth factor receptor signaling on tumor cells. The biological activity of anti-HER-2 antibodies has been observed in both animal models and in patients (26, 40, 41).

Problems with the long-term use of immunotoxins include limitations stemming from the antigenicity of the complex itself. However, there are numerous potential strategies that may be used to overcome these problems. For example, central to the problem with the therapeutic use of immunotoxins is the antigenicity of the antibody and the antigenic determinants on the toxin. However, the replacement of murine antibodies with mouse:human chimeric antibodies or the use of fully human antibodies can substantially reduce the problem (42, 43). Despite this, antigenic regions on the toxin itself can still be problematic. To eliminate this problem, molecular engineering studies to design potentially less immunogenic toxins are under way as are studies to replace plant toxins with less antigenic human cytotoxic cytokines. Initially, as part of this effort, in vitro and animal model studies were performed in our laboratory (44, 45) to identify the specific and potent chemical constructs of antibodies containing recombinant human TNF. These constructs, like those containing gelonin, appeared to be specifically toxic to antigen-presenting cells that are resistant to free TNF. Xenograft model studies have confirmed that antibody-TNF constructs specifically localize within tumor targets, can slow the growth of s.c. nodules, and can extend the survival of mice in a metastatic model. Additional studies in our laboratory have also identified gene fusion constructs of human TNF and single-chain antibodies recognizing HER-2/neu (46). Becker et al. (47) and Gillies et al. (48) have also designed and tested recombinant antibodies that recognize targets on human melanoma cells and that contain interleukin 2. Therefore, these studies may point the way in the development of recombinant constructs containing cytotoxic agents with reduced antigenicity that can be administered multiple times over a more prolonged period than is now possible.

In summary, we have shown that a construct of a recombinant human chimeric antibody BACH-250 recognizing HER-2/neu and containing rGel toxin binds to breast cancer cells expressing the HER-2/neu proto-oncogene. The binding and subsequent cytotoxic effects of the BACH-250/rGel immunotoxin were directly proportional to the cell-surface expression levels of HER-2. Tissue distribution and pharmacokinetic studies in human tumor xenograft models showed that the BACH-250/rGel exhibits plasma clearance kinetic characteristics and efficient tumor localization properties similar to those exhibited by the original BACH-250 antibody. Studies of the efficacy of the BACH-250/rGel immunotoxin against HER-2-expressing tumor xenografts showed that the BACH-250/rGel immunotoxin can suppress s.c. tumor growth and thereby increase the survival of mice in a lethal model.

ACKNOWLEDGMENTS

We acknowledge Donna Jares and Julia Merchant for their key role in the preparation of the manuscript.
**Fig. 13** BALB/c athymic mice (seven to eight mice/group) were implanted (day 0) s.c. with SKOV-3 tumor cells. Treatments were administered i.p. three times per week for 3 weeks starting on day 5 after implantation (tumor volumes were 0–30 mm³). Mice received either PBS (control), TAB-250 (42 μg/dose) plus gelonin (42 μg/dose), or the TAB-250/rGel conjugate (50 μg/dose). Tumors were measured twice per week with vernier calipers, and volumes (mm³) were calculated as a product of length × width × height. Tumor volumes were plotted as a function of day of growth. As shown in Fig. 10, TAB-250/rGel treatment enhanced the survival of mice with i.p. SKOV-3 tumors by an average of 12.4 days as compared with survival in mice treated with unconjugated TAB-250 or TAB-250 plus gelonin.

**REFERENCES**


Recombinant Immunotoxins Directed against the c-erb-2/HER2/neu Oncogene Product: In Vitro Cytotoxicity, Pharmacokinetics, and in Vivo Efficacy Studies in Xenograft Models

Michael G. Rosenblum, Laura K. Shawver, John W. Marks, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/4/865

Cited articles
This article cites 46 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/4/865.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/5/4/865.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.