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A Single-Chain IL-12 IgG3 Antibody Fusion Protein Retains Antibody Specificity and IL-12 Bioactivity and Demonstrates Antitumor Activity

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IL-12 is a heterodimeric cytokine with many actions on innate and cellular immunity that may have antitumor and antimetastatic effects. However, systemic administration of IL-12 can be toxic. Tumor-specific Abs provide a means to selectively target a metastatic/residual nodule and deliver therapeutic quantities of an immunostimulatory molecule like IL-12 with lower systemic levels and ideally, toxicity. We report the construction and characterization of an Ab fusion protein in which single-chain murine IL-12 is fused to an anti-Her2/neu Ab at the amino terminus (mscIL-12.her2.IgG3). The use of single-chain IL-12 in the fusion protein simplifies vector construction, ensures equimolar concentrations of the two IL-12 subunits, and may confer greater stability to the fusion protein. SDS-PAGE analysis shows this 320-kDa protein is secreted and correctly assembled. FACS analysis demonstrates that this fusion protein binds to cells transfected with the Her2/neu Ag, thus retaining Ab specificity; this fusion protein also binds to a cell line and to PHA-activated PBMC that express the IL-12R, thus demonstrating cytokine receptor specificity. T cell proliferation assays and NK cytotoxicity assays demonstrate that this fusion protein exhibits IL-12 bioactivity comparable to recombinant murine IL-12. In vivo studies demonstrate that this fusion protein has antitumor activity. These results are significant and suggest that this IL-12 Ab fusion protein can effectively combine the therapeutic potential of IL-12 with the tumor-targeting ability of the Ab and may provide a viable alternative to systemic administration of IL-12. The Journal of Immunology, 1999, 163: 250–258.

The management of residual and metastatic disease is a central problem in the treatment of cancer. Chemotherapeutic strategies can be effective, but are frequently limited by various toxicities. Therefore, additional modalities are needed to achieve disease containment or elimination. One approach has been to attempt to elicit a specific immune response by the host against tumor-associated Ags. Treatment with cytokines has been shown to render some nonimmunogenic tumors immunogenic, activating a protective immune response (1–4). However, when cytokines are given systemically there are frequently problems with toxicity that make it impossible to achieve an effective dose at the site of the tumor (5, 6). Ideally, strategies which increase the cytokine concentration at the site of the tumor and allow for lower systemic levels should be more effective.

Two approaches to achieving high levels of cytokine at the site of the tumor have been direct injection of cytokine into the tumor or transfer of the gene encoding the cytokine into tumor cells (7). While both methods have been shown to be effective, they also have significant limitations: direct injection into micrometastases is not possible, and currently gene transfer involves ex vivo manipulation of tumor cells, which makes treatment of large numbers of patients difficult and costly. Abs provide an alternative specific delivery vehicle in which tumor-specific Abs can be used to selectively target a metastatic/residual nodule and deliver an immunostimulatory molecule like a cytokine. The specific targeting should make it possible to elicit a systemic tumor-specific immune response without accompanying systemic toxicity.

There are many different types of tumor-associated Ags: oncofetal Ags (e.g., carcinoembryonic Ag (8)), Ags expressed on cells at a particular stage of differentiation (e.g., IL-2R (9)), growth factor receptors (e.g., transferrin receptor (10)), oncogene products (e.g., c-myc (11)), and the Id expressed by the surface Ig of lymphoma cells (12). These tumor-associated Abs distinguish normal from tumor tissue and have been used as targets for cancer therapy (13–17). Her2/neu, also known as c-erbB-2, is a cell surface oncogene product that is amplified and/or overexpressed in 25–30% of human breast and ovarian cancers with this overexpression associated with poor prognosis (18, 19). Humanized anti-Her2/neu has been shown to be an effective therapeutic agent in clinical trials (20). These trials demonstrate that metastatic breast cancer can be effectively targeted through the Her2/neu Ag and suggest that Abs specific for Her2/neu would be effective vehicles for targeting cytokines to the sites of the tumors.

Several different cytokines are attractive candidates for enhancing tumor-specific immune responses. IL-2 induces the proliferation of T cells, supports the growth of Ag-specific T cell clones, and enhances the activity of T and NK cells (21). Fusion of IL-2 to Abs specific for tumor-associated Ags such as ganglioside GD2 and the Id of a murine lymphoma has resulted in fusion proteins that have shown much promise as agents for stimulating tumor-specific immune responses (22–27). Indeed, an IL-2-Ab fusion protein specific for GD2 was able to generate an immune response
that eliminated metastatic disease in a murine model of melanoma (22).

Another cytokine that has great potential for use in tumor immunotherapy is IL-12. IL-12 is a heterodimeric cytokine with many actions on innate and cellular immunity that may have antitumor and antimetastatic effects. IL-12 can activate T and NK cells, induce the production of IFN-γ, and stimulate naive CD4+ T cells to differentiate toward the Th1 phenotype (28, 29). A Th1 response involves the secretion of a cytokine profile that activates cytotoxic T cells and macrophages, which could be desirable in an antitumor immune response. In addition, IL-12 may act through nitric oxide to cause cell-cycle arrest of tumor cells (4), and through induction of inducible protein-10 to inhibit angiogenesis (30).

Bioactive IL-12 requires the expression of two separate genes, p40 and p35, and correct heterodimer assembly (31). To address this issue, Gillyes et al. have recently reported the construction of an Ab-IL-12 fusion protein in which the p35 subunit was fused to the carboxyl terminus of an Ab; the p40 subunit was expressed as a separate polypeptide that must then assemble with the p35 subunit. Although this IL-12/Ab fusion protein was functional, the IL-12 bioactivity was 2-fold lower than rIL-12 (32). An alternative that eliminates the need to assemble two independently produced peptides is to express IL-12 as a single chain with the p40 and p35 subunits joined by a flexible linker. We have now used this alternative approach and constructed an Ab fusion protein in which murine single-chain (msc) IL-12 (p40 linker:p35) is fused to an anti-Her2/neu Ab at the amino terminus of the H chain (mscIL-12.her2.IgG3). Importantly, this fusion protein retains Ab specificity, exhibits IL-12 bioactivity comparable to recombinant murine (m) IL-12, and demonstrates antitumor activity in vivo.

Materials and Methods

Cell lines and reagents

P3X63Ag.8.653 cells (American Type Culture Collection, Manassas, VA), CT26 cells (murine colon adenocarcinoma cells kindly provided by Young Chul Sung, Pohang University, Korea), and CT26/Her2 cells (developed in our laboratory by transfection of CT26 cells with the cDNA encoding Her2/neu using methods previously described (33)) were cultured in IMDM supplemented with 5% bovine calf serum, L-glutamine, penicillin, and streptomycin. K562 cells (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% FBS, sodium pyruvate, and streptomycin. rIL-12.p40.linker.VEH, reference standard was kindly provided by Chiron, Emeryville, CA. rmIL-12 was kindly provided by Paul Carter, Genentech, South San Francisco, CA (34, 35) and cloned as previously described (36) into mammalian expression vectors (linearized with EcoRI). Transfected cells were plated at 2 x 10^4 cells/well in a flat-bottom 96-well tissue culture plate and selected with the addition of 10 mM histidinol (Sigma, St. Louis, MO) on days 3 and 5 after transfection. Wells were screened for Ab secretion after 10–14 days by ELISA using 96-well flat-bottom plates coated with goat anti-human IgG (Zymed, South San Francisco, CA). Supernatant from the transfected cells was applied, followed by the addition of goat anti-human κ conjugated with alkaline phosphatase (Sigma). Binding was detected by the addition of phosphatase substrate (para-nitrophenyl phosphate, disodium; Sigma), and positive wells were expanded.

To determine the size and assembly pattern of the secreted recombinant mscIL-12/her2.IgG3 Ab, supernatants from cells grown overnight in medium containing [35S]methionine (Amersham, Piscataway, NJ) were immunoprecipitated with polyclonal rabbit anti-human IgG and rabbit anti-human κ (produced by Letitia A. Wims in our laboratory), followed by staphylococcal protein A (IgGisorb; The Enzyme Center, Malden, MA). Immunoprecipitated Abs were analyzed on SDS-polyacrylamide gels in the presence or absence of reducing agent (2–ME).

For the purification of mscIL-12/her2.IgG3, high producing clones were expanded in roller bottles in IMDM plus 1% fetal clone serum plus Glutamax (Life Technologies, Rockville, MD), and cell-free culture supernatant was collected. Culture supernatants were passed through a protein A column; the column was washed with 10 ml PBS, and the proteins were successively eluted with 2 ml of 1 M citric acid, pH 4.5, 5 ml of 0.1 M glycine, pH 2.5, and 2 ml of 0.1 M glycine, pH 2.0. The eluted fractions were neutralized immediately with 2 M Tris-HCl, pH 8.0. The fractions were concentrated using Ultra-free-15 filters (Millipore, Bedford, MA) with a cut-off of 100 kDa and dialyzed. Using this method, 2 L of culture supernatant yields ~0.8 mg mscIL-12/her2.IgG3.

Assays of binding to Her2/neu Ag and IL-12R

Ag binding. CT26 or CT26/Her2 were incubated with mscIL-12/her2.IgG3, her2.IgG3, or dalsy1.IgG3 (an IgG3 isotype control Ab specific for the hapten dalsy for 1 h at 4°C. The cells were washed and incubated 2 h at 4°C with PE-labeled goat anti-human IgG (PharMingen, San Diego, CA) and analyzed by flow cytometry. Analysis was performed with a FACSscan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

Persistence of Ab binding at the cell surface. CT26/Her2 cells were incubated with mscIL-12/her2.IgG3, her2.IgG3, or dalsy1.IgG3. The cells were washed and incubated at 37°C in culture medium. At different time points (0, 1, 4, and 24 h), an aliquot of cells was removed and stained with PE-conjugated anti-human IgG followed by FACS analysis. The mean fluorescence was calculated as a percentage of the maximum mean fluorescence at time zero.

Binding to IL-12R. Kit255/K6 cells, a subclone of the human T leukemic cell line that expresses the IL-12R (40), were incubated with her2.IgG3 or mscIL-12/her2.IgG3. Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis. In a second assay, PHA-activated PBMC were incubated with her2.IgG3 or mscIL-12/her2.IgG3. PBMC have been shown to express IL-12Rs following activation with PHA and IL-2 (41). Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis.

Proliferation assays

Proliferation assays were performed as previously described (42). PBMC were isolated from normal blood donors by Ficoll-Hypaque density centrifugation (Ficoll-Paque, Pharmacia, Piscataway, NJ). These cells were then depleted of monocytes by plastic adherence, and nonadherent cells

3 Abbreviations used in this paper: msc, murine single-chain; m, murine; h, human; sc, single-chain; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PMS, phenazine methosulfate; PI, propidium iodide.
were resuspended at 5 × 10^7 cells/ml in supplemented medium [1:1 complete RPMI 1640:complete DMEM plus 5% human AB serum (Irvine Scientific, Santa Ana, CA), 10 mM HEPES, 0.0065% (w/v) L-arginine monohydrate, and 0.1% (w/v) dextrose] containing 2 μg/ml PHA-P (Difco Laboratories, Detroit, MI) and were cultured for 3 days. Cells were then split 1:1 with fresh supplemented medium containing 20 IU/ml rhIL-2 (kindly provided by Chiron Corporation) and incubated for a further 24–48 h. The PHA blasts were then washed with acidified RPMI 1640, pH 6.4, and rested in RPMI 1640 plus 0.5% human AB serum for 3–4 h. The cell concentration was adjusted to 2 × 10^6 cells/ml in supplemented medium. Neutralizing anti-IL-2 Ab (BioSource International, Camarillo, CA) was added at 1 μg/ml to block IL-2-induced proliferation.

Serial 1:3 dilutions of equivalent protein concentrations of mIL-12, mscIL-12.her2.IgG3, and her2.IgG3 were made in supplemented medium over a range of 36 ng/ml to 16 pg/ml. Next, 50 μg/ml rmIL-12 at 5 ng/ml, an equivalent IL-12 concentration of mscIL-12, or an equivalent Ab concentration of her2.IgG3 were added to the PBMC and incubated for 16–18 h at 37°C, 5% CO₂. The cell concentration was then adjusted to 0.5–1 × 10^6 cells/ml. Target K562 cells (2 × 10^4) were washed two times with serum-free RPMI 1640, then resuspended in 1 ml Diluent C (Sigma). Then, 4 μM PKH67 was prepared by diluting the stock solution (Sigma) in Diluent C. The cell suspension and dye were mixed in equal volumes (1 ml each) in a propylene tube and incubated at room temperature for 2 min. An equal volume (2 ml) of FBS was added to stop the labeling reaction. The cells were washed three times with RPMI 1640 plus 10% FBS and resuspended at 1–2 × 10^6 cells/ml in RPMI 1640 plus 10% FBS.

For the NK cytotoxicity assay, 100 μL effector PBMC and 100 μL PKH67-labeled K562 cells were added to polystyrene 12 × 75 mm tubes to create E:T ratios of 50:1 and 100:1 and were incubated for 4 h at 37°C, 5% CO₂. At the end of the incubation, 0.5 μl immunoprecipit (P1) at 5 μg/ml (Sigma) was added to each tube and immediately analyzed by FACS. Sponaneous cell death was determined by incubating either targets or effectors alone. FACS analysis was performed with a FACScan (Becton Dickinson) equipped with a blue laser excitation of 15 mW at 488 nm. The two fluorochromes, PKH67 and PI, were electronically compensated using PKH67-labeled targets alone and unstained target cells whose membrane had been permeabilized by treatment with 0.1% Tween-20 in PBS for 10 min at 37°C. These cells were then washed twice and 0.5 μl of isonicotinic acid added before FACS analysis. Data were collected in list mode and analyzed using Cell Quest software (Becton Dickinson). At least 2000 target events were collected per sample. Percent cytotoxicity was calculated as (number of dead targets)/total number of targets) × 100.

In vivo antitumor activity

A total of 1 × 10^6 CT26/Her2 cells in 0.15 ml PBS were injected s.c. into the right flank of syngeneic BALB/c mice on day 0. One group of mice was treated i.v. with mscIL-12/her2.IgG3 (at a concentration equivalent to 1 μg IL-12/day), her2.IgG3 (at a concentration equivalent to the Ab concentration of mscIL-12/her2.IgG3 administered/day), or PBS for 5 days beginning on day 1. A second group of mice was similarly treated beginning on day 6. In each group, 10 mice per treatment arm were used. Tumor growth was monitored and measured with a caliper every other day beginning on day 6 and continuing until day 20. At that point, all mice were euthanized and the tumors were harvested and weighed.

Results

Design and expression of single-chain (sc) IL-12 Ab fusion protein

The construction of her2.IgG3 and vectors for the production of H chain fusion proteins was previously described (36). For the present studies, we elected to use mIL-12 in our fusion protein because mIL-12 is biologically active on activated murine and human T and NK cells, while murine T and NK cells do not respond to hIL-12 (44). mscIL-12 was amplified from plasmid pSP72.mIL-12.p40.linker.Dp35 (kindly provided by Richard Mulligan) and joined to a (Gly₄Ser)₃ linker located at the amino terminus of the VH region of the her2.IgG3 Ab.

Enhanced NK activity of PBMC

These assays were performed according to the methods of Hatam et al. (43) with modifications. Briefly, effector PBMC were isolated as described above, then resuspended in RPMI 1640 plus 10% FBS at 1–10^6 cells/ml. rmIL-12 at 5 ng/ml, an equivalent IL-12 concentration of mscIL-12/her2.IgG3, or an equivalent Ab concentration of her2.IgG3 were added to the PBMC and incubated for 16–18 h at 37°C, 5% CO₂. The cell concentration was then adjusted to 0.5–1 × 10^6 cells/ml. Target K562 cells (2 × 10^4) were washed two times with serum-free RPMI 1640, then resuspended in 1 ml Diluent C (Sigma). Then, 4 μM PKH67 was prepared by diluting the stock solution (Sigma) in Diluent C. The cell suspension and dye were mixed in equal volumes (1 ml each) in a propylene tube and incubated at room temperature for 2 min. An equal volume (2 ml) of FBS was added to stop the labeling reaction. The cells were washed three times with RPMI 1640 plus 10% FBS and resuspended at 1–2 × 10^6 cells/ml in RPMI 1640 plus 10% FBS.

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her2.IgG3 does not appear to alter the assembly and secretion of the H₂L₂ form of the Ab.

Ag binding and persistence of Ab binding at the cell surface

The ability of mscIL-12.her2.IgG3 to bind to the Her2/neu antigenic target was examined using flow cytometry. Both mscIL-12.her2.IgG3 (Fig. 3B) and her2.IgG3 (Fig. 3C) specifically bound to CT26/Her2; neither Ab bound to parental CT26 cells (data not shown). Importantly, the same fluorescence intensity was seen with both her2.IgG3 and mscIL-12.her2.IgG3, suggesting that both have similar affinity for Her2/neu. A control IgG3 Ab specific for the hapten dansyl did not bind to CT26/Her2 (Fig. 3A). These data indicate that the fusion of a 75-kDa scIL-12 to the amino terminus of each H chain of her2.IgG3 does not interfere with the ability of the Ab to recognize the Her2/neu Ag.

There is no significant difference in the persistence of Ab bound to the cell surface between mscIL-12.her2.IgG3 and her2.IgG3, with both still showing similar fluorescence intensity at all time points and ≥30% staining at 24 h (Fig. 4). These results indicate that fusion of the Ab with IL-12 does not affect the dissociation rate, internalization, or degradation of mscIL-12.her2.IgG3 at the surface of the cell compared with her2.IgG3. This suggests that the IL-12 in our fusion protein will be present at the cell surface to activate T and NK cells.

Binding to the IL-12R

The ability of mscIL-12.her2.IgG3 to bind to the IL-12R was determined by flow cytometry of both transformed and normal human cells. The mscIL-12.her2.IgG3 bound to Kit225/K6, a subclone of the human T leukemic cell line that expresses the IL-12R (40), while her2.IgG3 did not (Fig. 5A). Neither her2.IgG3 or...
mscIL-12.her2.IgG3 bound to the resting PBMC (Fig. 5B). The mscIL-12.her2.IgG3 bound to the PHA-activated PBMC while her2.IgG3 did not (Fig. 5C). These results show that the IL-12 in the fusion protein is able to bind to the IL-12R.

**Proliferation assays**

After establishing that mscIL-12.her2.IgG3 was correctly assembled, secreted, and retained the ability to bind both the Her2/neu Ag and the IL-12R, we investigated its biologic activity. All assays of IL-12 biological activity were expressed relative to the IL-12 concentration used (i.e., ng/ml). To obtain the IL-12 concentration of the fusion protein, the fraction of the IL-12-Ab fusion protein that was Ab (170 kDa/320 kDa) was multiplied times the protein concentration of the fusion protein. In this way, the biological activity of IL-12 and IL-12 in the fusion protein could be compared on a per molecule basis. Similarly, to ensure that equivalent Ab concentrations of her2.IgG3 and mscIL-12.her2.IgG3 were used, the fraction of the Ab-fusion protein that was Ab (170 kDa/320 kDa) was multiplied times the protein concentration of the fusion protein to obtain the Ab concentration of the fusion protein. The same concentration of her2.IgG3 was then used as a control.

One of the pleiotropic actions of IL-12 is the ability to induce the proliferation of PHA-activated lymphoblasts. We prepared PHA-activated PBMC and incubated them for 48 h with mIL-12, mscIL-12.her2.IgG3, or her2.IgG3. Proliferation was measured by addition of MTS/PMS. Fig. 6 shows the results from a typical assay. mIL-12 and mscIL-12.her2.IgG3 showed an equivalent mitogenic effect on PHA-blasts in a dose-dependent manner. The results are expressed as the mean ± SD of triplicate samples with the background proliferation in medium subtracted. In contrast, her2.IgG3-treated PHA-blasts did not show any proliferation. These results indicate that the mitogenic effect of mscIL-12.her2.IgG3 is due to the IL-12 and not to some other effect by the Ab component of the fusion protein.

**Enhanced NK activity**

IL-12 has been shown to enhance the cytotoxic action of NK cells. We prepared PBMC (shown by FACS to be 8–9% CD56+; data not shown) and incubated them for 16–18 h with 5 ng/ml mIL-12 or an equivalent IL-12 concentration of mscIL-12.her2.IgG3. The PBMC were also incubated with her2.IgG3 at the same Ab concentration as mscIL-12.her2.IgG3 or medium. These effector cells were added to PKH67-labeled K562 target cells at E:T ratios of 100:1 and 50:1, then incubated for 4 h. After this incubation, PI, which intercalates into the DNA of dead cells, was added and FACS analysis performed. Fig. 7A shows a representative FACS result (E:T of 100:1, treated with 5 ng/ml mIL-12) with defined populations of live effectors (lower left), dead effectors (upper left), live targets (lower right), and dead targets (upper right) separated into the four quadrants. The x-axis measures PKH67 fluorescence intensity and the y-axis measures PI fluorescence intensity. A gate was drawn around target cells (PKH67 positive). Figs. 7, B–E show histograms of PI fluorescence intensity among target cells gated as in Fig. 7A. More positively staining cells (right) are dead target cells; less positive cells (left) are live target cells. The percent cytotoxicity was calculated from the histograms. The background cytotoxicity was calculated from the histograms. The background cytotoxicity in medium was subtracted to give the percent enhanced cytotoxicity. Fig. 7E shows that mIL-12 and mscIL-12.her2.IgG3 at equivalent IL-12 concentrations of 5 ng/ml and E:T of both 50:1 and 100:1 comparably enhanced NK cytotoxicity by ~20%, while her2.IgG3 showed no enhancement. These results indicate that the enhanced cytotoxicity by mscIL-12.her2.IgG3 is due to the IL-12 component of the fusion protein and not to some other effect by the Ab component of the fusion protein and that the IL-12 in the fusion protein has activity comparable to rIL-12.

**In vivo antitumor activity**

After demonstrating that mscIL-12.her2.IgG3 had in vitro biologic activity comparable to rIL-12, the in vivo antitumor activity was investigated using a CT26/Her2 animal model developed in our laboratory (33).

On day 0, CT26/Her2 cells were injected s.c. into the right flank of BALB/c mice. One group of mice was treated with mscIL-12.her2.IgG3, her2.IgG3, or PBS injected i.v. for 5 days beginning...
on day 1, while a second group was similarly treated beginning on day 6 when the tumors averaged 8–9 mm in diameter. Thus, the studies were designed to examine the effect of mscIL-12.her2.IgG3 on both tumor growth and tumor regression. Treatment with mscIL-12.her2.IgG3 slowed the growth of tumors when it began on day 1 (Fig. 8A) and arrested tumor growth when it began on day 6 (Fig. 8C) compared with mice treated with PBS or her2.IgG3. The tumor weights were used as a more objective indicator of tumor size and confirm the results of the caliper measurements (Fig. 8B and D).

These results demonstrate that mscIL-12.her2.IgG3 has significant antitumor activity in immunocompetent mice. Further studies are in progress to determine whether this effect can be seen in other tumor models and to determine the mechanism of the observed antitumor activity.

Discussion

In these studies, we describe the construction and expression of a novel bioactive mscIL-12 IgG3 Ab fusion protein. In the design of our Ab-IL-12 fusion protein, a number of factors were considered. Although our long-term goal is the production of Ab fusion proteins for therapeutic use in humans, mIL-12 was used for these initial studies because it has activity on both human and murine cells, while hIL-12 has activity only on human cells. The use of mIL-12 makes it possible not only to carry out assays using human PBMC to test biologic activity, but also to perform in vivo studies using immunocompetent mice to examine the effects against Her2/neu-expressing murine tumors.

Previous studies suggested that an accessible N terminus of the p40 subunit is important for IL-12 bioactivity. When Lieschke et al. constructed a scIL-12, the order of the subunits was found to affect the IL-12 biologic activity (45). When the p35 subunit came before the p40 subunit, there was greatly decreased IL-12 activity; in contrast, when the subunits were reversed, with p40 in front of p35, the scIL-12 had biologic activity comparable to rIL-12 (45–47). Similarly, in an OVA-IL-12 fusion protein in which the p40 subunit was fused to OVA, a 50-fold lower IL-12 activity was observed (48). Constraint of the p40 subunit in a fusion protein may disrupt the interaction between IL-12 and the IL-12R. The IL-12R complex consists of two chains, \( \beta_1 \) and \( \beta_2 \), with \( \beta_1 \) necessary for hIL-12 signaling and activity (49). It is thought that IL-12 interacts with the hIL-12R \( \beta_1 \) primarily through domains on the p40 subunit (50).

Given the need for an accessible p40 subunit, we chose to fuse the scIL-12 to the amino terminus of the H chain. We were concerned that if we fused the mIL-12.p40.linker.\( \Delta p35 \) to the carboxyl terminus of the Ab H chain, we would constrain the p40 subunit and lose IL-12 activity. In previous studies, it was found that both nerve growth factor (51) and B7.1 (36) had to be joined to the amino terminus of the Ab to maintain their activity in Ab fusion proteins; fusion at the carboxyl terminus of the H chain resulted in impaired activity in both cases. We find the IL-12 in our Ab-IL-12...
fusion protein to be fully functional with IL-12 bioactivity comparable to rIL-12.

Our studies contrast with the work of Gilles et al., who fused the p35 subunit to the carboxyl terminus of the H chain and expressed the p40 subunit from a separate vector (32). While this approach led to the production of functional fusion proteins, the IL-12 had only one-half of the expected bioactivity. In contrast to the single-chain approach, this approach requires the separate transfection of the two IL-12 subunits and does not guarantee that they are present in equimolar concentrations. Although the p40 subunit was not fused to the Ab in Ab-IL-12 fusion protein produced by Gilles et al., the fusion of the p35 subunit to the carboxyl terminus of the Ab without any type of flexible linker may make the p40 subunit somewhat less accessible for receptor binding; this could explain the 2-fold lower IL-12 activity they observed.

Both scIL-12 (75 kDa) and H chain (60 kDa) are large. However, by providing a flexible linker between the two polypeptides, we were able to maintain the activity of both. The presence of IL-12 at the amino terminus of the V\text{H} region does not sterically hinder the ability of the combining site of the Ab to interact with Ag on the cell surface and remain bound (see Figs. 3 and 4). Similarly, the IL-12 in the fusion protein appears to be unaffected in its ability to bind the IL-12R and exhibit IL-12-mediated cellular activation (Figs. 5–7). The attachment of the IL-12 to the V region of the Ab should position it near the surface of the tumor cell and may further potentiate the antigenicity of the targeted tumor.

The ultimate goal of the construction of mscIL-12.her2.IgG3 is its use as an antitumor agent. Using a CT26/Her2 tumor model previously developed in our laboratory (33), our initial in vivo studies demonstrate that this fusion protein has significant antitumor activity in immunocompetent BALB/c mice (Fig. 8). We observed better antitumor activity when treatment was started after the tumors were established with a mean diameter of 8–9 mm than when treatment was started the day after inoculation with tumor cells. This lends support to previous studies by others (4, 52, 53) in which better antitumor activity of IL-12 was observed when tumors were established. They proposed that this may be because effector cells are first recruited to the tumor site and are then activated by IL-12. Further work is being conducted to determine whether the in vivo efficacy we have observed is due to activated T or NK cells, whether a Th1 response has been stimulated, and whether any other antitumor activities may have been stimulated by treatment with mscIL-12.her2.IgG3.

In conclusion, we have demonstrated that it is possible to genetically engineer and express a scIL-12-Ab fusion protein that retains Her2/neu Ag specificity and IL-12 biologic activity comparable to rIL-12. Our results indicate that the bulky size of IL-12 does not affect Ag binding and that the Ab does not hinder cytokine receptor binding. Further, this fusion protein demonstrates
antitumor activity in a tumor model using CT26/Her2 cells in syngeneic immunocompetent BALB/c mice. Thus, this Ab-IL-12 fusion protein may be an effective alternate to systemic administration of IL-12 for the treatment of metastatic breast cancer. Using a tumor-targeting ability of the Ab, it should be able to achieve effective local IL-12 concentration at the sites of tumors and metastases with lower doses of IL-12, thus decreasing the risk of toxicity associated with IL-12 treatment. An anti-Her2/neu mAb has had success in clinical trials for the treatment of Her2/neu-expressing metastatic breast cancer (20). Fusion of a cytokine-like IL-12 to an Ab that has antitumor and antimetastatic properties to a Her2/neu-specific Ab may enhance its efficacy, particularly if it elicits a tumor-specific immune response.

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References