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The provision of the T cell costimulatory molecule B7 to tumor cells can be an effective means of triggering a tumor specific cytolytic T cells response. One way to provide B7 to tumor cells would be to couple an anti-tumor antibody either directly to B7 or to an antibody to the B7 counterreceptor on T cells, CD28. To this end, a fusion protein has been developed which incorporates a single chain antibody fragment (scFv) to erbB-2 (Her2/neu), an oncogene product overexpressed by 30-50% of breast carcinomas, and the extracellular domain of B7.2 (CD86). This fusion protein was expressed and purified from *Pichia pastoris*, shown to retain binding activity to both counter receptors, erbB-2 and CD28, and shown to provide the costimulatory signal to T cells through CD28. Thus, our fusion protein was shown capable of targeting erbB-2 postive tumor cells and delivering a CD28 specific T cell costimulatory signal. Further studies should characterize the fusion protein in erbB-2 tumor bearing mice for *in vivo* tumor targeting, biodistribution, and efficacy.

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PI - Signature   Date

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1. INTRODUCTION

In the initial proposal, two hypotheses were proposed for the development of fusion proteins designed to target erbB-2 bearing tumors and provide the costimulatory signal to anti-tumor T cells. One of the hypotheses proposed the development of bispecific scFv incorporating anti-CD28 scFv. In the last annual report we described the isolation and characterization of scFv specific for the T cell costimulatory receptor CD28 from a phage library. Unfortunately, these scFv failed to localize to CD28 bearing cells and thus were not utilized for development of bispecific scFv.

Because previous experiments had shown that the simple provision of B7 molecules to tumor cells by transfection could result in T cell activation and induce an anti-tumor T cell response, it was thought that incorporation of the extracellular domain of the B7 molecule into a fusion protein would be adequate, but perhaps not superior to anti-CD28 scFv, for costimulating T cells. Therefore, to determine whether provision of T cell costimulatory molecules to tumors by antibody based targeting was a viable alternative to tumor transfection, a fusion protein was proposed for development which incorporates the extracellular domain of the T cell costimulatory molecule, human B7.2 (hB7.2), separated by a flexible linker from an anti-erbB-2 scFv, C6ML3-9.B1 (B1).

2. BODY OF REPORT

2.1 Results

As a means of providing B7 to tumor cells for the activation of tumor specific T cells, a chimeric fusion protein was engineered and produced which can target erbB-2 expressing tumors and provide costimulation to T cells. The resulting fusion protein, hB7.2/B1, incorporated a high affinity scFv, C6ML3-B1, specific for erbB-2 (K_d = 120pM) and the extracellular domain of the CD28 ligand, human B7.2, separated by a polyglycine and serine linker.

In last year's report, the hB7.2/B1 chimeric fusion protein produced in Pichia pastoris, was shown to bind to both recombinant and cell surface tumor marker erbB-2 and to T cell counter receptor. HB7.2/B1 retained an extremely high affinity for erbB-2 showing a K_d of 580pM as compared to 120pM for B1 scFv (1).

For the hB7.2/B1 fusion protein to function as envisioned, it not only has to bind to both erbB-2 bearing tumor cells and to CD28 on T cells but also must provide an effective costimulatory signal to T cells for their activation to occur. To determine whether the hB7.2/B1 fusion protein could provide the costimulatory signal to T cells, costimulation

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assays were performed utilizing Jurkat T cells and latex beads coated with various combinations of antibodies and hB7.2/B1 fusion protein. First, sulfate polystyrene latex microspheres were coated overnight with various antibodies including anti-CD3, anti-CD28, and hB7.2/B1. The total concentration of antibody coated to latex beads was normalized with irrelevant Fv fragment. Following a 16-20 hour incubation of the coated latex beads with Jurkat T cells, IL-2 produced by the activated Jurkat cells was measured by IL-2 ELISA.

The results showed a strong IL-2 response occurred when Jurkat cells were stimulated with the positive control of anti-CD3 plus anti-CD28 coated latex beads as compared to anti-CD3 beads or cells alone (Figure 3.1). When anti-CD3 and hB7.2/B1 fusion protein were coated together and tested, the response was approximately 50% of that for the positive control, but still significantly greater than anti-CD3 beads or cells alone. IL-2 production by the Jurkat cells could be inhibited when stimulating with anti-CD3 plus hB7.2/B1 coated beads by addition of CTLA-4Ig or anti-hB7.2, indicating that the hB7.2 portion of the hB7.2/B1 fusion protein was providing the costimulatory signal to the T cells.

To test whether the hB7.2/B1 fusion protein could both target erbB-2 bearing cells and still effectively costimulate T cells, an alternative assay format was employed. In these assays, CHO-erbB-2 transfectants were preincubated with hB7.2/B1 for 1 hour and then added to Jurkat cells instead of latex beads and assays carried out in an identical manner as described above. CHO cells transfected with human B7.2 were used as a positive control. In all cases, PMA and ionomycin were used at 1 ng/ml and 1 uM, respectively, to augment the signal.

The combination of CHO-erbB-2 plus fusion protein induced an IL-2 response over four times that of CHO-c-erbB-2 alone and approximately two-thirds that of the positive control CHO-hB7.2 (Figure 3.2). In addition, the IL-2 produces by Jurkat T cells could be tittered over a range of hB7.2/B1 concentrations.

2.2 Conclusions

As a means of providing B7 to tumor cells for the activation of tumor specific T cells, a chimeric fusion protein was engineered and produced which can target erbB-2 expressing tumors and provide costimulation to T cells. The resulting fusion protein, hB7.2/B1, incorporated a high affinity scFv, C6ML3-B1, specific for erbB-2 (K_d = 120pM) and the extracellular domain of the CD28 ligand, human B7.2, separated by a polyglycine and serine linker.

For the proposed fusion protein to function as envisioned, an important assumption was made. If each half of the chimeric molecule is available for binding, and the orientation of the molecule is appropriate for simultaneous binding to erbB-2 on tumor cells and to CD28 on T cells, then the fusion protein should be able to induce activation and proliferation of T cells with similar benefits as were seen with B7 transfected tumor cells (2-8). The orientation of the fusion protein, that is whether the T cell costimulatory molecule is at the N-terminus or the C-terminus, was not originally considered to be of primary importance. Initially, a fusion protein was constructed with hB7.2 at the C-terminus of the molecule, however binding experiments with the molecule in this orientation showed a lack of functionality of hB7.2. Another group's work supported this observation. Challita-Eid et al. showed that hB7.1 does not retain native binding characteristics when incorporated into a fusion protein at the C-terminus (9). Subsequent construction of the chimeric molecule with hB7.2 at the N-terminus resulted in a more successful molecule.

The hB7.2/B1 chimeric fusion protein produced in *Pichia pastoris*, was shown to bind to both recombinant and cell surface tumor marker erbB-2 and to T cell counter receptor. HB7.2/B1 retained an extremely high affinity for erbB-2 showing a K_d of 580pM as compared to 120pM for B1 scFv (1). When hB7.2/B1 was either coated on latex beads or bound to CHO cells transfected with erbB-2, the CD28 binding entity was available to bind T cells and provided costimulation resulting in increased IL-2 production by T cells over control. Therefore, both components of the hB7.2/B1 chimeric fusion protein are independently functional and, in addition, hB7.2/B1 can bind both counter receptors simultaneously. The hB7.2 component is also able to provide the costimulatory signal to T cells resulting in increased activation. Therefore, our chimeric fusion protein appears to function as envisioned and may be suitable for further *in vivo* testing.

There are theoretical advantages to activating T cells via costimulatory molecules as compared to anti-CD3 or other conjugates. The three main advantages can be summed up as increased specificity, increased range of targets, and longevity of the therapeutic effect. Firstly, T cell activation via costimulatory molecules is antigen specific; the non-tumor killing associated with either CD3 crosslinking, general cytokine therapy, or immunotoxin or radioisotope conjugates would be prevented. For example, in anti-tumor therapy using an anti-erbB-2 antibody coupled to CD3, all cells expressing erbB-2 would be targeted for destruction, whereas only those cells displaying immunogenic peptides in the context of MHC molecules should be targeted when using hB7.2/B1. In addition, the severe toxicity associated with the use of immunotoxins or radioisotope antibody conjugates would be avoided. Secondly, tumor cells need not express erbB-2 to be targeted. Provided that

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immunogenic peptides displayed by MHC molecules are common between erbB-2 expressers and non-expressers, once a response is generated against a particular antigen, T cells should respond to that antigen regardless of the presence of the erbB-2 marker (5). The importance of this point should not be understated; even tumor cells that lose expression of the target marker can still be destroyed. A final advantage of activation via costimulatory molecules is the induction of protective immunity. Recurrences should not occur because the immune system will be primed for further responses to tumor growth.

Recently, two reports have been published which describe the construction and characterization of fusion proteins combining an anti-erbB-2 antibody and either hB7.1 or hB7.2 (9, 10), thereby supporting our hypothesis. Gestmayer *et al.* constructed a chimeric molecule incorporating hB7.2 and a murine derived scFv. In contrast, Challita-Eid *et al.* utilized hB7.1 and a human IgG3 Ab containing murine variable domains. The orientation of these fusion proteins is similar in that the costimulatory molecule was engineered at the N-terminus of the molecule. Both groups showed that their chimeric fusion proteins localize to erbB-2 bearing cells and provide the costimulatory signal to T cells, resulting in increased T cell proliferation.

The chimeric fusion protein reported here, hB7.2/B1, has several distinct advantages over the previously published molecules mentioned above. First, the anti-erbB-2 entity of hB7.2/B1 has an extremely high affinity for the tumor antigen. B1 scFv is one of the highest affinity scFv produced to date by any means (K_d = 120 pM) and therefore should display better tumor targeting, specificity, and retention than other chimeric molecules targeting erbB-2 with lessor affinities. In comparison to hB7.2/B1, Challita-Eid *et al.* reported that their fusion protein displayed an affinity for immobilized erbB-2 of 170nM (9). Second, the entire sequence of the hB7.2/B1 fusion protein is of human origin. A disadvantage of murine antibodies or antibody fragments is that they are likely to be immunogenic when administered therapeutically. Murine or chimeric IgG are clearly immunogenic when administered to humans and some of the immune response is directed against the variable regions (11). Therefore, chimeric fusion proteins produced by other groups containing murine scFv or murine variable regions may be less attractive for human administration. Finally, the size of the B7 fusion partner is important for tumor penetration. Fusion proteins containing full-length antibody molecules instead of scFv may have decreased tumor retention times and overall efficacy because of limited tumor access.

In summary, a chimeric fusion protein was engineered and characterized for tumor targeting and T cell costimulation. The hB7.2/B1 chimeric fusion protein was shown to function as envisioned and should be analyzed further in erbB-2 tumor bearing mice for tumor targeting, biodistribution, and efficacy. It is hoped that the outcome of *in vivo*

experiments may provide information useful in either improving the hB7.2/B1 fusion protein for further testing or in the design of new molecules that may eventually succeed in the eradication of cancerous disease.

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FIGURE 3.1. T Cell Costimulation Utilizing Latex Beads Coated with HB7.2/B1.

[IL-2]ng/ml

FIGURE 3.2. T Cell Costimulation Utilizing CHO-erbB-2 Transfectants and HB7.2/B1.

[IL-2]ng/ml

Reagents Added
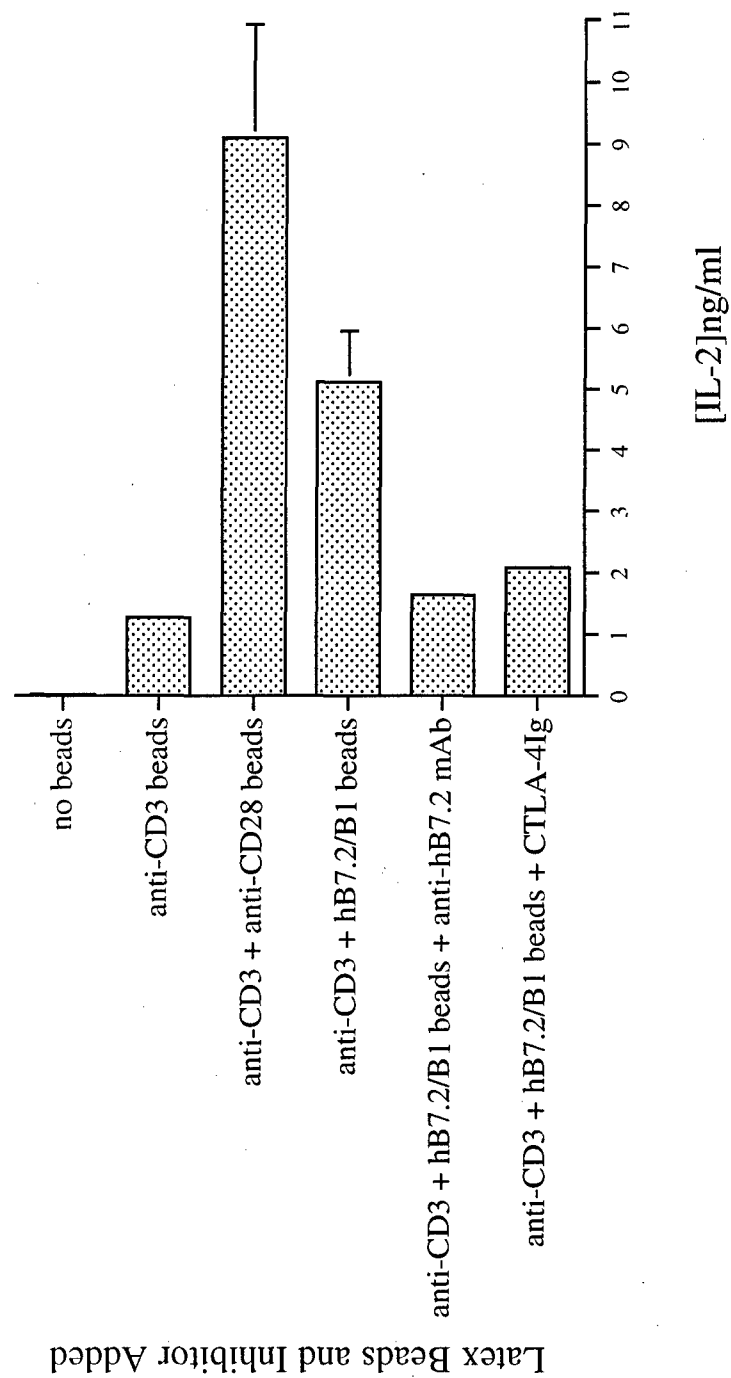
nothing added
CHO-erbB-2
anti-CD28 mAb
CHO-hB7.2
CHO-erbB-2 + 10ug/ml hB7.2/B1
CHO-erbB-2 + 1ug/ml hB7.2/B1
CHO-erbB-2 + 0.1ug/ml hB7.2/B1
CHO-erbB-2 + 0.01ug/ml hB7.2/B1
CHO-erbB-2 + 0.001ug/ml hB7.2/B1
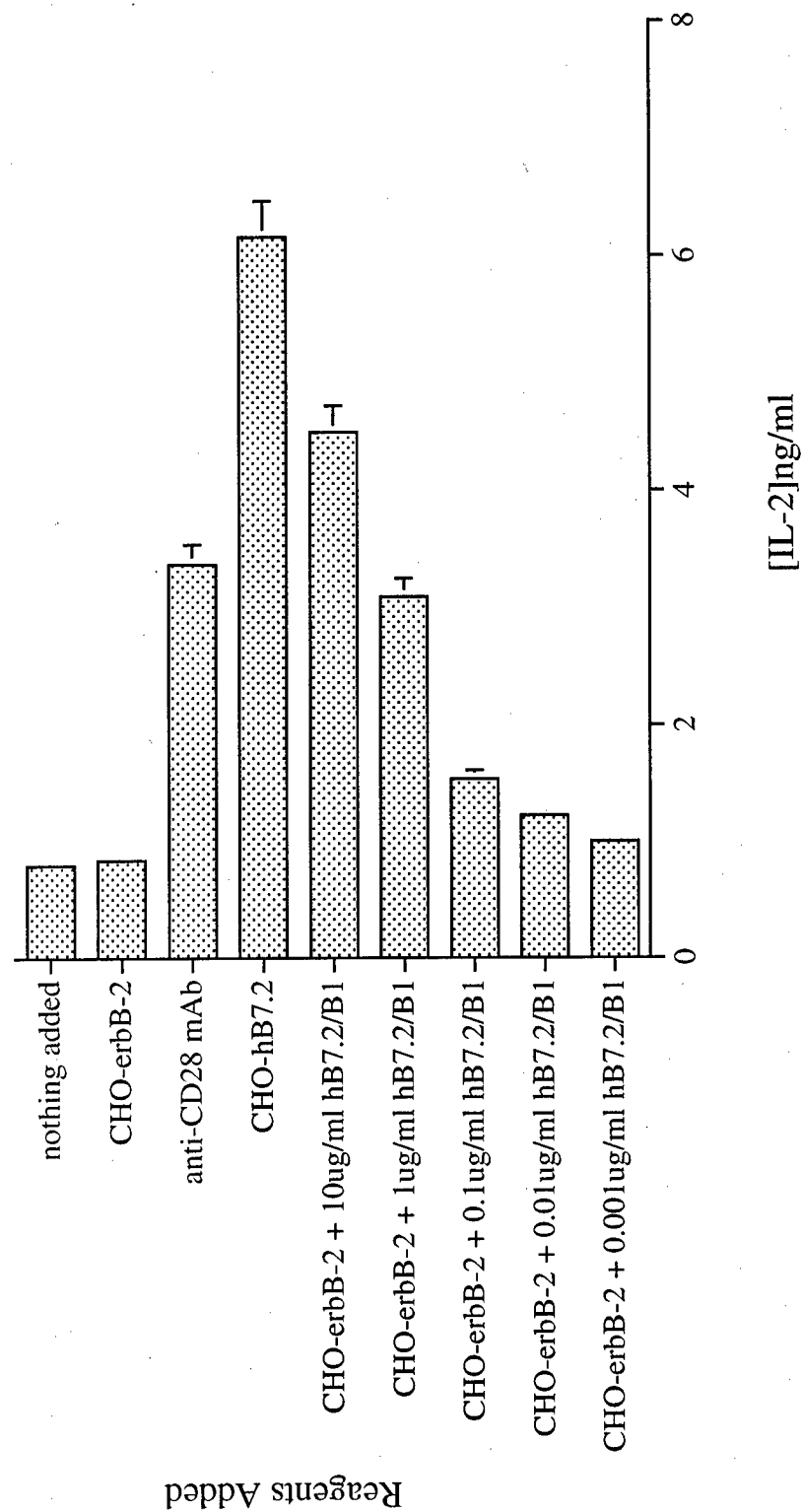
3.3 Figure Legends

<u>FIGURE 3.1. T Cell Costimulation Utilizing Latex Beads Coated with HB7.2/B1.</u>

IL-2 ELISA were performed to measure the IL-2 production by Jurkat T cells incubated for 16-20 hours with sulfate polystyrene latex microspheres coated with various combinations of antibody and fusion protein. In some cases, the addition of 5 ug/ml CTLA-4Ig or 1 ug/ml anti-hB7.2 antibody was included as an inhibitor of costimulation.

<u>FIGURE 3.2. T Cell Costimulation Utilizing CHO-erbB-2 Transfectants and HB7.2/B1.</u>

IL-2 ELISA were performed to measure the IL-2 production by Jurkat T cells incubated for 16-20 hours with CHO-erbB-2 transfectants pre-incubated with hB7.2/B1. The plot shows the reagents that were added to Jurkat T cells and the corresponding quantity of IL-2 produced. CHO cells stably transfected with hB7.2 cDNA were used as a positive control as was the addition of 10 ug/ml anti-hCD28 mAb. In all cases, 1 ng/ml PMA and 1 uM ionomycin were added to augment the signal.

4 APPENDICES

4.1 Key Research Accomplishments

- genetic engineering, expression, and purification of the hB7.2/B1 chimeric fusion protein.
- determination of binding properties of the hB7.2/B1 chimeric fusion protein to immobilized and cell surface counter receptor.
- determination of T cell costimulatory ability of the hB7.2/B1 chimeric fusion protein.
- initiation of *in vivo* characterization experiments on the hB7.2/B1 chimeric fusion protein.

4.2 Reportable Outcomes

An abstract and poster describing this work was included in the DoD BCRP "Era of Hope" conference in the fall of 1997. They were entitled, "Engineering of Novel Breast Cancer Immunotherapeutics Targeting Cytolytic T Cells."

A manuscript entitled, "Engineering and Characterization of a Novel Fusion Protein Incorporating B7.2 and an Anti-ErbB-2 ScFv for the Activation of Cytotoxic T Cells," has been prepared and is currently being reviewed by faculty members to determine where it should be submitted.

The degree of Ph.D. in Pharmaceutical Chemistry is pending approval of the dissertation based on work supported by this award.

4.3 Copy of Manuscripts and Abstracts

An abstract and poster describing this work was included in the DoD BCRP "Era of Hope" conference in the fall of 1997. They were entitled, "Engineering of Novel Breast Cancer Immunotherapeutics Targeting Cytolytic T Cells."

ABSTRACT

The human immune system does not mount an effective attack against breast cancer cells, suggesting that one or more factors required for antigen specific activation of cytolytic T cells is absent. Recent experiments indicate that the provision of the B7 costimulatory molecule to the tumor cell surface can result in activation of tumor specific cytolytic T cells with corresponding rejection of tumor cells.

One way to provide B7 to tumor cells would be to couple an anti-tumor antibody either directly to B7 or to an antibody to the B7 counterreceptor on T cells, CD28. To this end, a fusion protein has been developed which incorporates a single chain antibody fragment (scFv) to c-erbB-2 (Her2/neu), an oncogene product overexpressed by 30-50% of breast carcinomas, and the extracellular domain of B7-1 (CD80). Experiments are currently underway to determine if the fusion protein can bind to c-erbB-2 and CD28, and also to measure its ability to activate T-cells. To explore whether this fusion protein is effective *in vivo*, it will subsequently be tested for its ability to elicit an anti-tumor response to P815 mastocytomas in a mouse system.

In addition, single chain antibody fragments are currently being selected, using a phage display scFv library, which are specific for the T cell costimulatory receptor molecule CD28. Anti-CD28 scFv with a range of affinities will be isolated and the role of affinity and differential binding to T cell costimulatory receptors will be determined to identify their importance in the biology of T cell activation. Since the affinity of B7 for CD28 has been estimated at 4uM, a higher affinity interaction to signal T cells through CD28 may be more advantageous. Anti-CD28 scFv with preferential binding to CD28 versus CTLA-4 may be more effective at T cell costimulation since CTLA-4 is thought to down-regulate T cell responses. Anti-CD28 scFv also can be used with anti-c-erbB-2 scFv to make bispecific antibodies for tumor therapy that may be more efficacious than fusion proteins utilizing B7.

ENGINEERING AND CHARACTERIZATION OF A NOVEL FUSION PROTEIN INCORPORATING B7.2 AND AN ANTI-ERBB-2 SINGLE CHAIN ANTIBODY FRAGMENT FOR THE ACTIVATION OF CYTOTOXIC T CELLS

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ABSTRACT

The provision of the T cell costimulatory molecule B7 to tumor cells can be an effective means of triggering a tumor specific cytolytic T cell response. One way to provide B7 to tumor cells would be to couple an anti-tumor antibody directly to B7. Such a molecule should target tumors displaying antigen and provide the costimulatory signal to T cells, resulting in the initiation of an anti-tumor T cell response. To this end, a fusion protein was designed which incorporates a single chain antibody fragment (scFv) to erbB-2 (Her2/neu), an oncogene product overexpressed by 30-50% of breast carcinomas, and the extracellular domain of B7-2 (CD86). This fusion protein, expressed and purified from *Pichia pastoris*, was shown to retain binding activity to both counter receptors, erbB-2 and CD28. The fusion protein was also shown capable of targeting erbB-2 positive tumor cells and delivering a CD28 specific T cell costimulatory signal. These results suggest that a fusion protein engineered to target tumor cells and signal T cells for activation may be an effective means of cancer immunotherapy. Further studies should be performed to characterize the fusion protein in erbB-2 tumor bearing mice for *in vivo* tumor targeting, biodistribution, and efficacy.

KEY WORDS: cancer immunotherapy, B7.2, erbB-2, single chain Fv, T cell costimulation

INTRODUCTION

Contact of the T cell receptor with MHC-peptide complexes is necessary but not sufficient for T-cell activation (1). If T cells receive the primary MHC-peptide signal through the TCR, but a second, costimulatory signal is not received, then T cells may enter a state of long-term unresponsiveness, leaving them ineffective at eliminating the target cells (2, 3). The most significant receptor/ligand pair that can provide the costimulatory signal is thought to be a member of the B7 family on antigen presenting cells (APC) interacting with CD28 on T cells (4, 5).

The observation that the human immune system does not mount an effective attack against tumor cells suggests that one or more factors required for an antigen specific immune response is not present. Since the expression of B7 molecules are generally limited to professional APC, it has been hypothesized that tumor cells lack the ability to provide the costimulatory signal to T cells, and thus cannot independently activate tumor specific T cells. Indeed, recent experiments support the concept that provision of either B7.1(CD80) or B7.2(CD86) to tumor cells can result in their elimination in a cytotoxic T lymphocyte (CTL) dependent manner (6-12). Several investigators have transfected murine tumor cells with either B7 molecules alone, or in combination with MHC molecules, and determined their effects on tumor cell growth *in vivo* (6-12). In general, tumor cells transfected with B7 molecules alone were shown to specifically activate CD8+ T cells, resulting in rejection of tumor cells *in vivo*. In some cases, T cell activation also resulted in protection against subsequent challenge with unmodified tumor cells. Human tumor cell lines transfected with B7 molecules were also able to stimulate in vitro T cell proliferation and cytotoxic T cell responses (13). These experiments suggest that provision of T cell costimulatory molecules to tumor cells can lead to tumor rejection.

Instead of transfecting tumor cells *ex vivo*, provision of T cell costimulatory molecules to tumor cells can be accomplished using antibody based targeting. Tumor specific antibody targeting requires identification of molecules whose expression is

generally limited to tumor cells, or that are at least overexpressed by tumors. One viable choice for tumor targeting of breast cancer is the oncogene product erbB-2, which has been demonstrated to be overexpressed in 30-50% of human breast carcinomas (14, 15).

To determine whether provision of T cell costimulatory molecules to tumors by antibody based targeting was a viable alternative to tumor transfection, a fusion protein was developed which incorporates an anti-erbB-2 scFv, C6ML3-9.B1 (B1), and the T cell costimulatory molecule, human B7.2 (hB7.2). The fusion protein proposed would be entirely human in sequence, would bind to erbB-2 expressing breast tumor cells with high affinity, and should be able to costimulate T cells by signaling through CD28.

MATERIALS AND METHODS

Antibodies and Recombinant Proteins

Antibodies used include anti-human B7.2 clone IT2.2, anti-human CTLA-4 clone BN13, anti-human CD28 clone CD28.2, anti-human CD3 clone UCHT1), rat anti-human IL-2 clone MQ1-17H12, and biotinylated mouse anti-human IL-2 clone B33-2 all from Pharmingen, San Diego, CA. Also used were anti-erb-B-2 antibody clone 4D5 (Paul Carter, Genentech, San Francisco, CA), anti-C6.5 polyclonal Ab serum, anti-myc tag clone 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA), FITC conjugated anti-mouse or anti-human antibodies (Sigma, St. Louis, MO), and anti-human B7.1 clone B7-24 (M. de Boer, PanGenetics, Amsterdam, Netherlands).

Recombinant proteins used include recombinant human IL-2 (Pharmingen, San Diego, CA), soluble CTLA-4Ig fusion protein (M. de Boer, PanGenetics, Amsterdam, Netherlands), and soluble human CD28Ig (Peter Linsley, Bristol-Myers Squibb, Seattle, WA).

Cell Lines

All cell lines were cultured at 37°C and 5% CO2 in a humidified incubator. In all cases, cell lines were maintained in medium supplemented with 10% FCS, 2mM glutamine, antibiotics and, additionally, G418 in the case of transfected lines. CHO and CHO-c-erbB-2 (provided by Dr. J. Rosenblatt, U. of Rochester, Rochester, NY) cell lines were grown in F12 medium. CHO-humanCTLA-4 stably transfected cells, a kind gift of Dr. U. Pessara (Boehringer Mannheim, Penzberg, Germany), were maintained in MEMα with deoxyribonucleosides (Life Technologies, Gaithersburg, MD). CHO-humanB7.2 stably transfected cells were generously provided by Dr. G. Freeman (Dana Farber Cancer Institute, Boston, MA) and were grown in F12/DMEM medium. Jurkat cells were obtained from Dr. A. Weiss (UCSF, San Francisco, CA) and were grown in complete RPMI. The *Pichia pastoris* yeast strain GS115 was utilized for protein expression and growth conditions are provided below (Invitrogen, San Diego, CA).

Construction of Fusion Protein Construct

The fusion protein construct was initially assembled in the scFv pHenIX vector, a derivative of the phagemid pHenI 16. The gene for human B7.2 was cloned into the VH site of pHenIX with Xho1 and Not1, whereas the entire anti-erb-B-2 scFv C6ML3.9-B1 (B1) was cloned into the VL site with ApaL1 and Not1. A human B7.2 gene vector, LL277 (kindly provided by Dr. L. Lanier, DNAX, Palo Alto, CA), was used for the PCR amplification of amino acids 1-225 of the extracellular domain of B7.2. The B7.2 5' primer contained a Xho1 restriction site (5'gggCTgACCTAggACTC gAgggAATgTggTCT gggggAggC-3') and the 3' primer contained a Nco1 restriction site (3'gTCCTCg CAACTCCATggCCgCTCCTCTgAAgATTCAAgC-5'). The scFv clone B1 was PCR amplified using primers containing the ApaL1 (5'gTCCTCgCAACT gAgTgCACAggTgCAgCTgTTgCAgTCTggg-3') and Not1 (3'-gggCTgACCTAggACgC ggCCgCggCCgCACCTAggACggTCAgC-5') restriction sites. Constructs were confirmed correct by DNA sequencing.

The entire construct was next subcloned using Sfi1 and Not1 into pPICZαB (Invitrogen, San Diego, CA), a *Pichia pastoris* expression vector containing both myc epitope and polyhistidine tags. Sufficient quantities of the plasmid vector containing the fusion protein construct were produced in the E. coli strain DH5α for Pme 1 linearization and transformation by electroporation into GS115 according to manufacturer's instructions (Invitrogen). The construct was again sequenced to confirm correctness.

Fusion protein expressing transformants were screened for optimal expression levels by performing small scale expressions and measuring protein expreesion levels by dot blot analysis and ELISA. Briefly, 96-well plates (Nunc; Maxisorp) were coated with 10ug/ml erbB-2 extra cellular domain (gift of Jim Huston, Creative Biomolecules) (17), blocked in 5% FCS/PBS, and incubated for four hours with Pichia expression supernatent. Bound fusion protein was detected with subsequent one hour incubations with each 9E10 anti-myc tag antibody and anti-mouse HRP. The HRP substrate, ABTS (Sigma, St. Louis, MO), was added and fluorescence read at 405nm in a fluorometer.

Large Scale Expression and Purification of Fusion Protein

Large scale expression was performed in shaker flasks at 30°C and 250rpm for 48 hours after induction in methanol. Briefly, 500 ml cultures of a high fusion protein expressing Pichia pastoris clone was grown overnight in BMGY plus Zeocin (50 mg/ml) and kanamycin (50 mg/ml) until the OD_{600} was 4-6. The cells were then diluted to an OD_{600} of 1.0 in 2000 mls of fresh BMMY media containing 50 mg/ml kanamycin to induce. Fresh methanol was added to 0.5 % to maintain induction at 24 hours. After 48 hours, the cells were removed by centrifugation and a protease inhibitor cocktail (Sigma, #P2714) was added to the supernatent to reduce proteolysis of the fusion protein.

For purification, medium containing the secreted fusion protein was concentrated and buffer exchanged by tangential flow in a Centramate system using a 30kD filter cassette

(Pall Gellman Sciences, Ann Arbor, MI). To minimize precipitation of salts during buffer exchange, 0.1M potassium phosphate buffer pH 6.0 was used to facilitate transfer into PBS pH 7.2. Fusion protein was purified by IMAC (18) and gel filtration on a Superdex S-200 column (Pharmacia) and appropriate fractions concentrated on Microsep 10K spin filters (Pall Gellman Sciences).

Post-translational modification was analyzed by treatment with PNGase. Fusion protein was denatured by 10 minutes at 100°C and incubated with PNGase overnight at 37°C. Both untreated and deglycosylated fusion protein was analyzed by SDS-PAGE and western blotting using anti-C6.5 polyclonal sera.

Recombinant Antigen Binding Assays

Recombinant antigen binding ELISA were performed with 10ug/ml erbB-2 (gift of Jim Huston, Creative Biomolecules), immobilized on 96 well microtiter plates by overnight incubation at 4°C. Plates were blocked for one hour at room temperature with 5% FCS/PBS and fusion protein was allowed to bind for 4 hours. Detection was made possible by addition of CTLA-4Ig or anti-B7 mAb for 1 hour and then HRP conjugated anti-human or anti-mouse Ab, followed by incubation with peroxidase substrate and measurement at 405nm in an ELISA plate reader.

Measurement of Affinity and Binding Kinetics by SPR in a BIAcore

The K_d of purified chimeric fusion protein was determined using surface plasmon resonance in a BIAcore (Pharmacia Biosensor). All experiments were performed at 37°C in HBSS buffer ((25 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20)). In a BIAcore flow cell, approximately 1400 resonance units (RU) of 90kDa erbB-2 ECD (10ug/ml in 10mM sodium acetate pH 4.5) were coupled to a CM5 sensor chip using NHS-EDC chemistry 19. Association rates were measured under continuous flow of 15ul/minute using concentrations ranging from 5.0×10^{-8} to 8.0 to 10^{-7}

M. k_{on} was determined from a plot of $(\ln(dR / dt))/t$ versus concentration. k_{off} was determined during from the dissociation part of the sensogram at the highest concentration of protein analyzed using a flow rate of 15ul/minute. K_d was calculated as k_{off} / k_{on}.

Cell Surface Binding Assays for Fusion Protein

Cell surface binding of the fusion protein was performed by FACS analysis on a FACScan (Becton Dickinson). Adherent cells were treated with Cell Dissociation Buffer (Life Technologies, Gaithersburg, MD), and washed two times in PBS. For binding to erbB-2, CHO-erb transfectants were incubated at 5×10^5 with either 0.1 or 1.0ug of fusion protein for 45 minutes at 4°C. Cells were washed twice and stained by incubation with either 9E10 or anti-B7 mAb and FITC labeled goat anti-mouse IgG (Sigma) for 30 minutes. As a positive control, cells were stained with 4D5 and anti-mouse FITC. For CTLA-4 binding, CHO-CTLA-4 transfectants were used and the combination of 9E10 and FITC labeled goat anti-mouse IgG utilized for detection. As a positive control, cells were stained with anti-CTLA-4 and anti-mouse FITC. In both cases the CHO wild type cell line was used as a control.

Costimulation Assays Using Latex Microspheres

Costimulation assays utilizing sulfate polystyrene latex microspheres were performed as has been previously described (20). Briefly, latex beads of 5 +/- 0.1um (Interfacial Dynamics, Portland, OR) were coated with fusion protein and/or other signaling molecules. Approximately 2×10^7 latex beads were coated overnight in 500ul PBS at 4°C with varying combinations of anti-CD3 (2ug), anti-CD28 (4ug), and fusion protein. The total concentration of antibody coated to latex beads was normalized with irrelevant Fv fragment to 5ug/ml. Following the overnight incubation, beads were blocked by one hour incubation with the addition of 500ul of 2%BSA/PBS and subsequently washed three times in 1%BSA/PBS to remove unbound antibody. Jurkat assays were

performed in 96 well culture plates by combining 2×10^5 Jurkat cells with varying quantities of coated latex beads (4×10^5 - 1.6×10^6) and incubating 20 hours in a tissue culture incubator. The following day, IL-2 production was measured by IL-2 ELISA using PharMingen protocol and antibodies.

IL-2 ELISA were performed in 96 well plates coated overnight with 4ug/ml anti-IL-2 antibody and blocked with 5% FCS/PBS. Medium was transferred from 96 well culture plates, in which Jurkat cells were exposed to coated latex beads, to the anti-IL-2 coated plates. After a four hour incubation at room temperature, plates were washed and additionally incubated for one hour with each of biotinylated anti-IL-2 and streptavidin HRP (Sigma, St. Louis, MO). The HRP substrate, ABTS, was added and fluorescence read at 405nm in a fluorometer. Quantitation of IL-2 produced by Jurkat cells was determined by comparing to a standard curve measured with recombinant human IL-2.

Costimulation Assays Using Transfected CHO Cells

For IL-2 assays using transfected CHO cells, 5×10^4 CHO-erb cells were preincubated with fusion protein or control antibody for 1 hour at 4°C in RPMI. Cells were then added to 96 well tissue culture plates with Jurkat cells and assays carried out as described above. PMA and ionomycin (IM) were used at 5ng/ml and 1uM, respectively (Sigma, St. Louis, MO). Anti-CD28 was used at 10ug/ml. Supernatents were harvested at 16-20 hours and IL-2 measured by ELISA.

RESULTS

Construction of the Human B7.2/Anti-erbB-2 Fusion Protein

A chimeric fusion protein was engineered containing both the extracellular domain (ECD) of human B7.2 (CD86) residues 1-225 and the anti-erbB-2 scFv, B1. The two proteins were connected by a polyglycine and serine linker (SSGGGGSGGGGSGGSA) to provide sufficient flexibility to allow both molecules to bind their respective counter

receptors simultaneously. Fusion proteins with hB7.2 at either the N- or C-terminus were produced but only at the N-terminus did hB7.2 retain native binding characteristics (data not shown). The observation that decreased affinity of a B7 molecule for CD28 when incorporated into the C-terminus of a fusion protein was also reported elsewhere 21.

The genetic construct for the chimeric fusion protein was assembled in the scFv pHenIX vector with the gene encoding for hB7.2 ECD residues 1-225 PCR amplified from a vector containing the full length gene (gift of Dr. Lewis Lanier, DNAX) and subcloned into the V_H site and the B1 scFv subcloned into the V_L site (Figure 3.1). The resulting construct, hB7.2/B1, encoded for a fusion protein with hB7.2 at the N-terminus and B1 scFv at the C-terminus. To produce hB7.2/B1 chimeric fusion protein, a yeast system was chosen for expression over *E. coli* because hB7.2 has been shown to be heavily glycosylated. The hB7.2/B1 construct was subsequently subcloned into the *Pichia pastoris* expression plasmid pPICZαB (Invitrogen) which contains the alcohol oxidase promoter (AOX1) for high level expression of heterologous proteins, the yeast α-factor signal sequence for protein secretion, and the myc epitope and $(His)_6$ tags for detection and purification, respectively (Figures 3.2 and 3.3).

Expression and Purification of the Chimeric Fusion Protein hB7.2/B1

Small scale expressions were initially performed to screen for good expressors of the fusion protein. In all cases, hB7.2/B1 was expressed in Pichia strain GS115 by inoculating BMGY media and growing overnight at 30°C. Cells were centrifuged and resuspended in BMMY media at pH 6.0 and grown for 24-72 hours at 30°C. To screen a number of small scale samples for expression, culture supernatents were tested by ELISA (described below). Large scale expressions were performed on the best expressing clones in 2 liter shaker flasks. To purify hB7.2/B1 from culture, cellular debris was removed by centrifugation and supernatent containing the secreted fusion protein was concentrated and

buffer exchanged by tangential flow in a Centramate system using a 30 kDa filter cassette (Pall Gellman Sciences, Ann Arbor, MI). HB7.2/B1 was purified by IMAC (18) and gel filtration on a Superdex S-200 column (Pharmacia) and appropriate fractions concentrated on Microsep 10K spin filters (Pall Gellman Sciences). Purified protein was analyzed by SDS-PAGE and Western blotting using polyclonal anti-C6.5 rabbit serum for detection (Figure 3.4). The fusion protein appeared highly glycosylated and ran as a smear between approximately 90-110 kDa instead of the calculated molecular weight of 57.3 kDa. When treated with PNGase, the molecular weight of the fusion protein was reduced to approximately 60 kDa. Two bands can be seen near this molecular weight corresponding to hB7.2/B1 fusion protein with or without the myc tag which has been observed to be cleaved from scFv over time.

Binding Assays on Immobilized Antigen

The ability of hB7.2/B1 to simultaneously bind both to the extracellular domain of erbB-2 and to the B7 receptor was determined by enzyme linked immunosorbent assay (ELISA). Briefly, ELISA were performed with the extra cellular domain (ECD) of erbB-2 (gift of Jim Huston, Creative Biomolecules) immobilized on 96 well plates. HB7.2/B1 fusion protein was allowed to bind and detected by addition of CTLA-4Ig (gift of Dr. Mark de Boer), anti-B7 mAb (PharMingen), or anti-myc tag 9E10 mAb, followed by HRP conjugated anti-human Fc or anti-mouse mAb, incubation with peroxidase substrate, and finally measurement of absorbance at 405nm. Typical results of such an assay are shown in Figure 3.5. For screening of clones to determine best expressors as described above, the anti-myc tag antibody was utilized for detection, indicating only that hB7.2/B1 was present and that the anti-erbB-2 scFv was functional. However, in the case of detecting with either CTLA4Ig or anti-B7 mAb, the results indicate that not only can each half of the hB7.2/B1 chimeric fusion protein bind to its respective counter receptor independently, but

also that the fusion protein can adopt an orientation conducive to binding of both counter receptors simultaneously.

Determination of Kinetics by Surface Plasmon Resonance

To determine whether the anti-erbB-2 scFv half of the fusion protein retained its affinity for immobilized antigen, kinetics were measured by surface plasmon resonance on a BIAcore. Briefly, erbB-2 ECD was immobilized on a CM5 sensor chip using NHS-EDC chemistry. Associations were measured under continuous flow of 15ul/minute using a range of concentrations from 800 nM to 50 nM. k_{on} was determined from a plot of $(\ln(dR/dt))/t$ versus concentration. k_{off} was determined during the first two minutes of dissociation of the scFv from erbB-2 ECD and K_d was calculated as k_{off}/k_{on}. The hB7.2/B1 fusion protein bound immobilized erbB-2 ECD with kinetics similar to those reported for the B1 scFv (Figure 3.6) with only a several fold difference in the K_d at 580 pM for hB7.2/B1 as compared to 120 pM for B1 scFv 22. Thus, the chimeric fusion protein retained extremely high affinity for targeting the erbB-2 tumor antigen.

HB7.2/B1 Fusion Protein Binds Specifically to Cells Bearing Counter Receptors

The ability of each half of the hB7.2/B1 fusion protein to bind to its respective immobilized counter receptor did not guarantee that hB7.2/B1 would be capable of binding to cell surface antigen. To this end, CHO cells transfected with either erbB-2 or CTLA-4 cDNA were used to determine whether hB7.2/B1 could bind to both tumor antigen and T cell costimulatory receptor.

Cell surface binding of the hB7.2/B1 fusion protein was performed by FACS analysis on a FACScan (Becton Dickinson). For binding to erbB-2, either cell lines CHO or CHO-erb transfectants (provided by Dr. J. Rosenblatt) were incubated at 5×10^5 with 1.0ug of fusion protein for 45 minutes at 4°C. Cells were washed twice and stained by

incubation with anti-hB7.2 mAb and FITC labeled goat anti-mouse IgG (Sigma) for 30 minutes. For CTLA-4 binding, parental CHO cells and CHO-CTLA-4 transfectants (provided by U. Pessara) were used and the combination of anti-myc tag antibody, 9E10, and FITC labeled goat anti-mouse IgG utilized for detection.

In both cases, binding of the hB7.2/B1 fusion protein to cell surface erbB-2 or CTLA-4 was measurable and gave good shifts in fluorescence as compared with untransfected CHO cells, demonstrating that hB7.2/B1 should be capable of targeting erbB-2 bearing tumor cells and also capable of binding to costimulatory molecules on T cells (Figure 3.7).

Fusion Protein Can Provide the Costimulatory Signal to T cells

For the hB7.2/B1 fusion protein to function as envisioned, it not only has to bind to both erbB-2 bearing tumor cells and to CD28 on T cells but also must provide an effective costimulatory signal to T cells for their activation to occur. To determine whether the hB7.2/B1 fusion protein could provide the costimulatory signal to T cells, costimulation assays were performed utilizing Jurkat T cells and latex beads coated with various combinations of antibodies and hB7.2/B1 fusion protein. First, sulfate polystyrene latex microspheres were coated overnight with various antibodies including anti-CD3, anti-CD28, and hB7.2/B1. The total concentration of antibody coated to latex beads was normalized with irrelevant Fv fragment. Following a 16-20 hour incubation of the coated latex beads with Jurkat T cells, IL-2 produced by the activated Jurkat cells was measured by IL-2 ELISA.

The results showed a strong IL-2 response occurred when Jurkat cells were stimulated with the positive control of anti-CD3 plus anti-CD28 coated latex beads as compared to anti-CD3 beads or cells alone (Figure 3.8). When anti-CD3 and hB7.2/B1 fusion protein were coated together and tested, the response was approximately 50% of that for the positive control, but still significantly greater than anti-CD3 beads or cells alone.

IL-2 production by the Jurkat cells could be inhibited when stimulating with anti-CD3 plus hB7.2/B1 coated beads by addition of CTLA-4Ig or anti-hB7.2, indicating that the hB7.2 portion of the hB7.2/B1 fusion protein was providing the costimulatory signal to the T cells.

To test whether the hB7.2/B1 fusion protein could both target erbB-2 bearing cells and still effectively costimulate T cells, an alternative assay format was employed. In these assays, CHO-erbB-2 transfectants were preincubated with hB7.2/B1 for 1 hour and then added to Jurkat cells instead of latex beads and assays carried out in an identical manner as described above. Soluble anti-CD28 mAb or CHO cells transfected with human B7.2 served as a positive controls. In all cases, PMA and ionomycin were used at 1 ng/ml and 1 uM, respectively, to augment the signal.

Results showed the combination of CHO-erbB-2 plus fusion protein induced an IL-2 response over four times that of CHO-c-erbB-2 alone and approximately two-thirds that of the positive control CHO-hB7.2 (Figure 3.9). In addition, the IL-2 produced by Jurkat T cells could be tittered over a range of hB7.2/B1 concentrations.

DISCUSSION

As a means of providing B7 to tumor cells for the activation of tumor specific T cells, a chimeric fusion protein was engineered and produced which can target erbB-2 expressing tumors and provide costimulation to T cells. The resulting fusion protein, hB7.2/B1, incorporated a high affinity scFv, C6ML3-B1, specific for erbB-2 (K_d = 120pM) and the extracellular domain of the CD28 ligand, human B7.2, separated by a polyglycine and serine linker.

For the proposed fusion protein to function as envisioned, an important assumption was made. If each half of the chimeric molecule is available for binding, and the orientation of the molecule is appropriate for simultaneous binding to erbB-2 on tumor cells and to CD28 on T cells, then the fusion protein should be able to induce activation and

proliferation of T cells with similar benefits as were seen with B7 transfected tumor cells 6-12. The orientation of the fusion protein, that is whether the T cell costimulatory molecule is at the N-terminus or the C-terminus, was not originally considered to be of primary importance. Initially, a fusion protein was constructed with hB7.2 at the C-terminus of the molecule, however binding experiments with the molecule in this orientation showed a lack of functionality of hB7.2. Another group's work supported this observation. Challita-Eid et al. showed that hB7.1 does not retain native binding characteristics when incorporated into a fusion protein at the C-terminus 21. Subsequent construction of the chimeric molecule with hB7.2 at the N-terminus resulted in a more successful molecule.

The hB7.2/B1 chimeric fusion protein produced in Pichia pastoris, was shown to bind to both recombinant and cell surface tumor marker erbB-2 and to T cell counter receptor. HB7.2/B1 retained an extremely high affinity for erbB-2 showing a K_d of 580pM as compared to 120pM for B1 scFv 22. When hB7.2/B1 was either coated on latex beads or bound to CHO cells transfected with erbB-2, the CD28 binding entity was available to bind T cells and provided costimulation resulting in increased IL-2 production by T cells over control. Therefore, both components of the hB7.2/B1 chimeric fusion protein are independently functional and, in addition, hB7.2/B1 can bind both counter receptors simultaneously. The hB7.2 component is also able to provide the costimulatory signal to T cells resulting in increased activation. Therefore, our chimeric fusion protein appears to function as envisioned and may be suitable for further *in vivo* testing.

To date, bispecific antibodies have been produced which trigger the immune system, but until recently few attempted to activate T-cells via a costimulatory signal. For example, bispecific antibodies have been generated which are composed of anti-tumor antibodies coupled to anti-CD3 (23, 24). A limitation of targeting CD3 alone, however, is that crosslinking of CD3 by bispecific antibodies results in T cell activation regardless of MHC expression, immunogenic peptide display, or T cell restriction. Other fusion proteins

which incorporate an anti-tumor antibody and cytokines are equally non-specific and could result in significant non-tumor killing.

There are theoretical advantages to activating T cells via costimulatory molecules as compared to anti-CD3 or other conjugates. The three main advantages can be summed up as increased specificity, increased range of targets, and longevity of the therapeutic effect. Firstly, T cell activation via costimulatory molecules is antigen specific; the non-tumor killing associated with either CD3 crosslinking, general cytokine therapy, or immunotoxin or radioisotope conjugates would be prevented. For example, in anti-tumor therapy using an anti-erbB-2 antibody coupled to CD3, all cells expressing erbB-2 would be targeted for destruction, whereas only those cells displaying immunogenic peptides in the context of MHC molecules should be targeted when using hB7.2/B1. In addition, the severe toxicity associated with the use of immunotoxins or radioisotope antibody conjugates would be avoided. Secondly, tumor cells need not express erbB-2 to be targeted. Provided that immunogenic peptides displayed by MHC molecules are common between erbB-2 expressers and non-expressers, once a response is generated against a particular antigen, T cells should respond to that antigen regardless of the presence of the erbB-2 marker (9). The importance of this point should not be understated; even tumor cells which lose expression of the target marker can still be destroyed. A final advantage of activation via costimulatory molecules is the induction of protective immunity. Recurrences should not occur because the immune system will be primed for further responses to tumor growth.

Recently, two reports have been published which describe the construction and characterization of fusion proteins combining an anti-erbB-2 antibody and either hB7.1 or hB7.2 (21, 25), thereby supporting our hypothesis. Gestmayer *et al.* constructed a chimeric molecule incorporating hB7.2 and a murine derived scFv. In contrast, Challita-Eid *et al.* utilized hB7.1 and a human IgG3 Ab containing murine variable domains. The orientation of these fusion proteins is similar in that the costimulatory molecule was engineered at the N-terminus of the molecule. Both groups showed that their chimeric

fusion proteins localize to erbB-2 bearing cells and provide the costimulatory signal to T cells, resulting in increased T cell proliferation.

The chimeric fusion protein reported here, hB7.2/B1, has several distinct advantages over the previously published molecules mentioned above. First, the anti-erbB-2 entity of hB7.2/B1 has an extremely high affinity for the tumor antigen. B1 scFv is one of the highest affinity scFv produced to date by any means (K_d = 120 pM 22) and therefore should display better tumor targeting, specificity, and retention than other chimeric molecules targeting erbB-2 with lessor affinities. In comparison to hB7.2/B1, Challita-Eid et al. reported that their fusion protein displayed an affinity for immobilized erbB-2 of 170nM (21),. Second, the entire sequence of the hB7.2/B1 fusion protein is of human origin. A disadvantage of murine antibodies or antibody fragments is that they are likely to be immunogenic when administered therapeutically. Murine or chimeric IgG are clearly immunogenic when administered to humans and some of the immune response is directed against the variable regions (26). Therefore, chimeric fusion proteins produced by other groups containing murine scFv or murine variable regions may be less attractive for human administration. Finally, the size of the B7 fusion partner is important for tumor penetration. Fusion proteins containing full length antibody molecules instead of scFv may have decreased tumor retention times and overall efficacy because of limited tumor access.

In summary, a chimeric fusion protein was engineered and characterized for tumor targeting and T cell costimulation. The hB7.2/B1 chimeric fusion protein was shown to function as envisioned and should be analyzed further in erbB-2 tumor bearing mice for tumor targeting, biodistribution, and efficacy. It is hoped that the outcome of in vivo experiments may provide information useful in either improving the hB7.2/B1 fusion protein for further testing or in the design of new molecules which may eventually succeed in the eradication of cancerous disease.

ACKNOWLEDGMENTS

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FIGURES

FIGURE 3.1. Map of ScFv pHenIX Vector.

The hB7.2/B1 fusion protein was originally constructed in the scFv pHenIX vector. HB7.2 ECD (1-225) was cloned into the VH site Xho1/Not1 and the B1 scFv was cloned into the VL site ApaL1/Not1. The resulting construct encodes for hB7.2 at the N-terminus of the protein, followed by a flexible linker (SSGGGGSGGGGSGGSA), and the B1 scFv at the C-terminus.

FIGURE 3.2. Map of pPICzalphaB.

A schematic diagramming the pPICzαB vector. The fusion protein construct assembled in the pHenIX vector was subcloned Sfi1/Not1 into the multiple cloning site of pPICzαB for expression in Pichia pastoris. The pPICzαB vector contains the myc and polyhistidine tags for detection and purification, respectively.

FIGURE 3.3. Sequence of the HB7.2 Chimeric Fusion Protein

The theoretical sequence of the hB7.2/B1 fusion protein as encoded for in the pPICzαB vector. Both the hB7.2 and B1 scFv portions of the fusion protein sequence are underlined and several other portions including the myc and polyhistidine tags are in bold.

FIGURE 3.4. Analysis of HB7.2/B1 Fusion Protein by SDS-PAGE and Western Blotting.

A. SDS-PAGE analysis of the hB7.2/B1 fusion protein. Lane M: Molecular weight markers (kilodaltons). Lane 1: Commassie stained hB7.2/B1 fusion protein purified from Pichia pastoris.

B. Western analysis of hB7.2/B1 fusion protein. Lane 1: Untreated hB7.2/B1. Lane 2: PNGase treated hB7.2/B1 to remove N-linked glycosylation (Lane 2). Lane M: Molecular weight markers (kilodaltons). HB7.2/B1 fusion protein was detected in blots by addition of anti-C6.5 polyclonal sera and HRP conjugated anti-rabbit mAb.

FIGURE 3.5. **Ability of HB7.2/B1 Fusion Protein to Bind to Counter Receptors as Determined by ELISA.**

The ability of hB7.2/B1 to bind to both immobilized erbB-2 and to soluble anti-B7 molecule was determined by ELISA. ErbB-2 ECD coated microtiter plates were incubated with either purified hB7.2/B1 or B1 scFv and binding detected with either CTLA-4Ig, hB7.2 mAb, hB7.1 mAb or control followed by HRP conjugated anti-human Fc mAb or anti-mouse Fc mAb. Absorbance was measured at 405nm on an ELISA plate reader.

FIGURE 3.6. **Kinetics of HB7.2/B1 as Measured by BIAcore Analysis.**

The binding kinetics of hB7.2/B1 to immobilized erbB-2 ECD were determined by surface plasmon resonance as measured on a BIAcore. The plot shows association and dissociation curves of purified hB7.2/B1 at varying concentrations. k_{on} was determined from a plot of $(\ln(dR / dt))/t$ versus concentration. k_{off} was determined during the dissociation part of the sensorgram at the highest concentration of protein analyzed using a flow rate of 15ul/minute. K_d was calculated as k_{off} / k_{on}.

FIGURE 3.7. **Cell Surface Binding of Fusion Protein to Transfected CHO Cells**

The ability of hB7.2/B1 fusion protein to bind to either erbB-2 or CTLA-4 on cell surfaces was determined by FACS analysis utilizing transfected CHO cells. A,C. The binding of hB7.2/B1 to the parental CHO cell line. B. The binding of hB7.2/B1 to CHO cells stably transfected with a human erbB-2 cDNA. D. The binding of hB7.2/B1 to CHO cells stably transfected with CTLA-4 cDNA. Detection in A and B was made using anti-hB7.2 antibody and for C and D using anti-myc antibody, 9E10, and in all cases with FITC labeled anti-mouse IgG.

FIGURE 3.8. **T Cell Costimulation Utilizing Latex Beads Coated with HB7.2/B1.**

IL-2 ELISA were performed to measure the IL-2 production by Jurkat T cells incubated for 16-20 hours with sulfate polystyrene latex microspheres coated with various combinations

of antibody and fusion protein. In some cases, the addition of 5 ug/ml CTLA-4Ig or 1 ug/ml anti-hB7.2 antibody was included as an inhibitor of costimulation.

FIGURE 3.9.   T Cell Costimulation Utilizing CHO-erbB-2 Transfectants and HB7.2/B1.

IL-2 ELISA were performed to measure the IL-2 production by Jurkat T cells incubated for 16-20 hours with CHO-erbB-2 transfectants pre-incubated with hB7.2/B1.   The plot shows the reagents that were added to Jurkat T cells and the corresponding quantity of IL-2 produced. CHO cells stably transfected with hB7.2 cDNA were used as a positive control as was the addition of 10 ug/ml anti-hCD28 mAb. In all cases, 1 ng/ml PMA and 1 uM ionomycin were added to augment the signal.
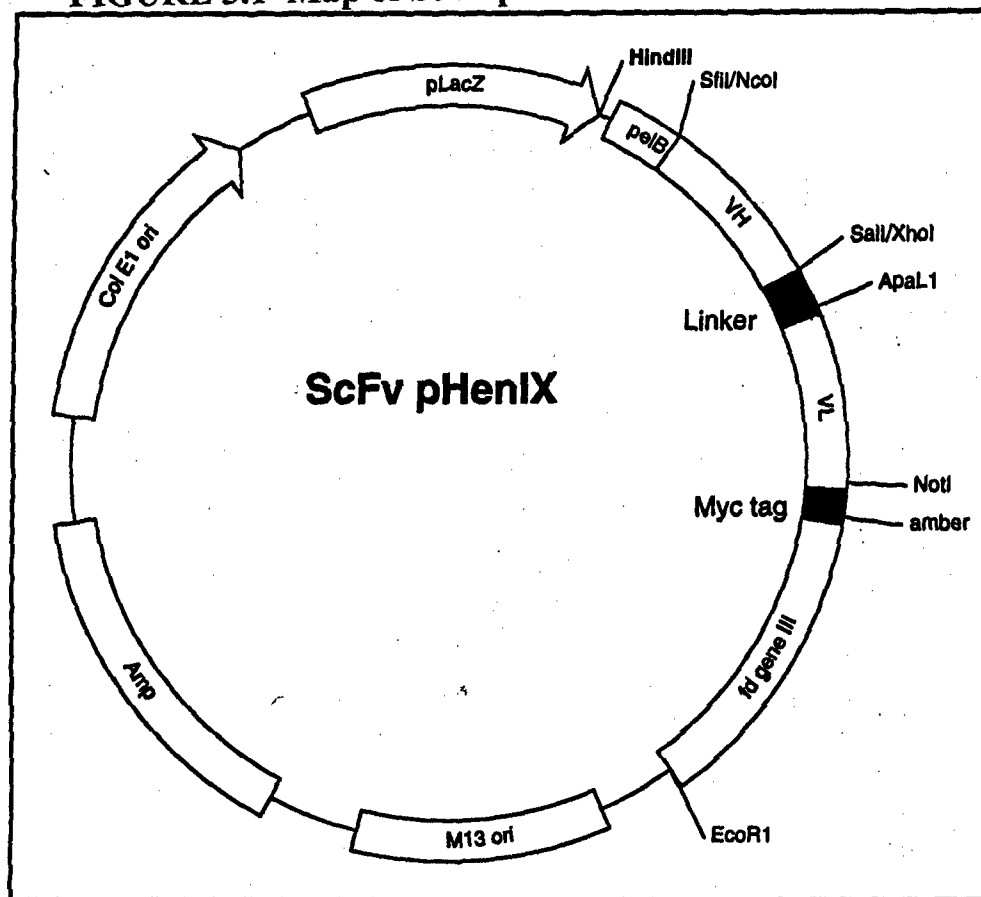
FIGURE 3.1 Map of ScFv pHenIX Vector

FIGURE 3.2. Map of pPICZalphaB

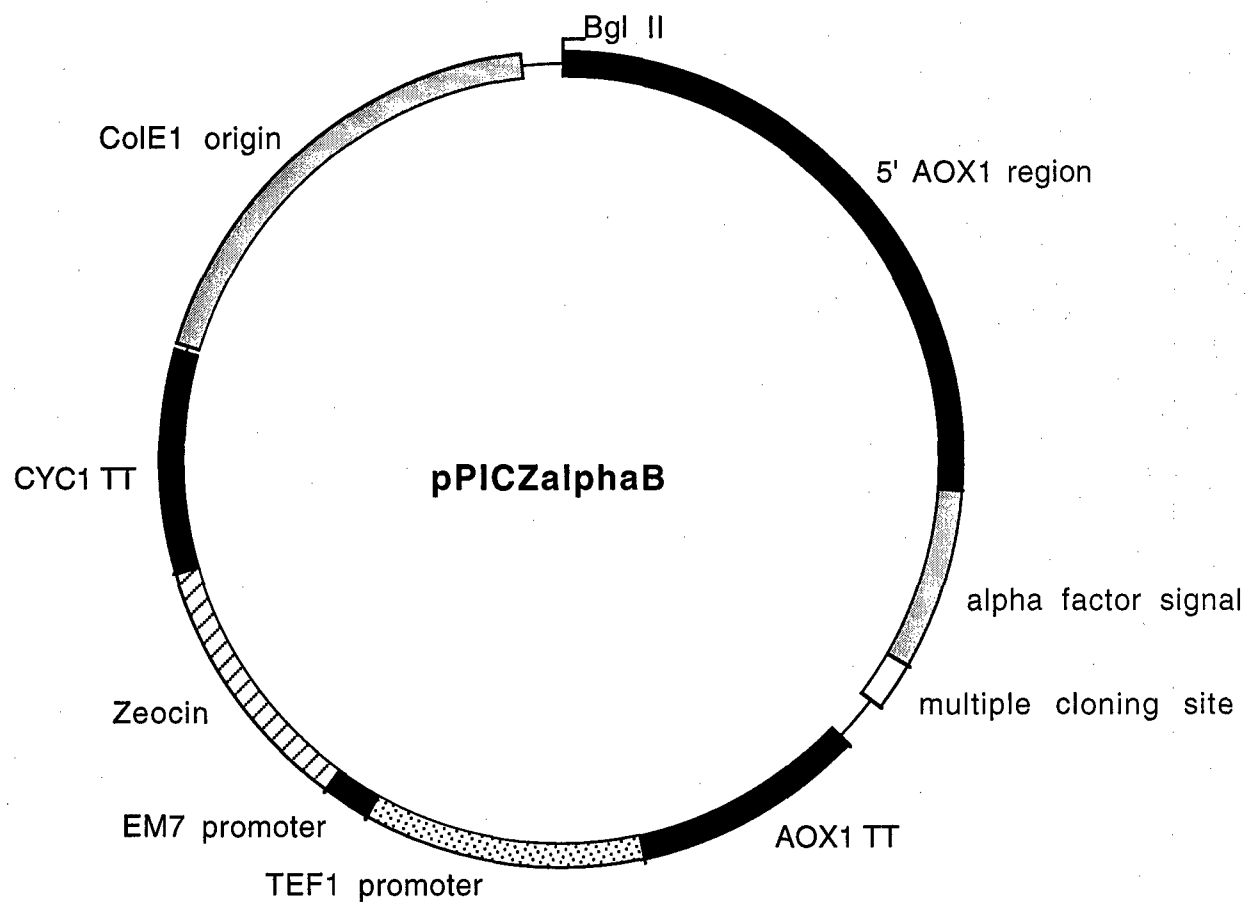**FIGURE 3.3. Sequence of the HB7.2/B1 Chimeric Fusion Protein**

1   70
AGIHVAQAPLKIQAYFNETADLPCQFANSQNQSLSELVVFWQDQENLVLNEVYLGKEKFDSVHSKYMGRT
polylinker   hB7.2 ECD (1-225)   hB7.2
71   145
SFDSDSWTLRLHNLQIKDKGLYQCIIHHKKPTGMIRIHQMNSELSVLANFSQPEIVPISNITENVYINLTCSSIH
                                                hB7.2
146   220
GYPEPKKMSVLLRTKNSTIEYDGIMQKSQDNVTELYDVSISLSVSFPDVTSNMTIFCILETDKTRLLSSPFSIEL
                                                hB7.2
221   290
EDPQPPPDHIP **SSGGGGGSGGGGSGGGSA** QVQLLQSGAELKKPGESLKISCKGSGYSFTSYWIAWVRQMPGK
                 linker   B1 scFv (1-258)
291   361
GLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDVGYCTDRTCAKWPE
                                                B1 scFv
362   432
YFQHWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSQSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLP
                                                B1 scFv
433   503
GTAPKLLIYGHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYCAAWDDSLSGWVFGGGTKLTVLGA
                                                B1 scFv
504   531
AAGASFL EQKLISEEDLN SAVD **HHHHHH**
            myc tag   his tag

FIGURE 3.4. Analysis of HB7.2/B1 Fusion Protein by SDS-PAGE and Western Blotting.

A.

M   1

200

97   -> hB7.2/B1

69

46

30

21.5

B.

1   2   M

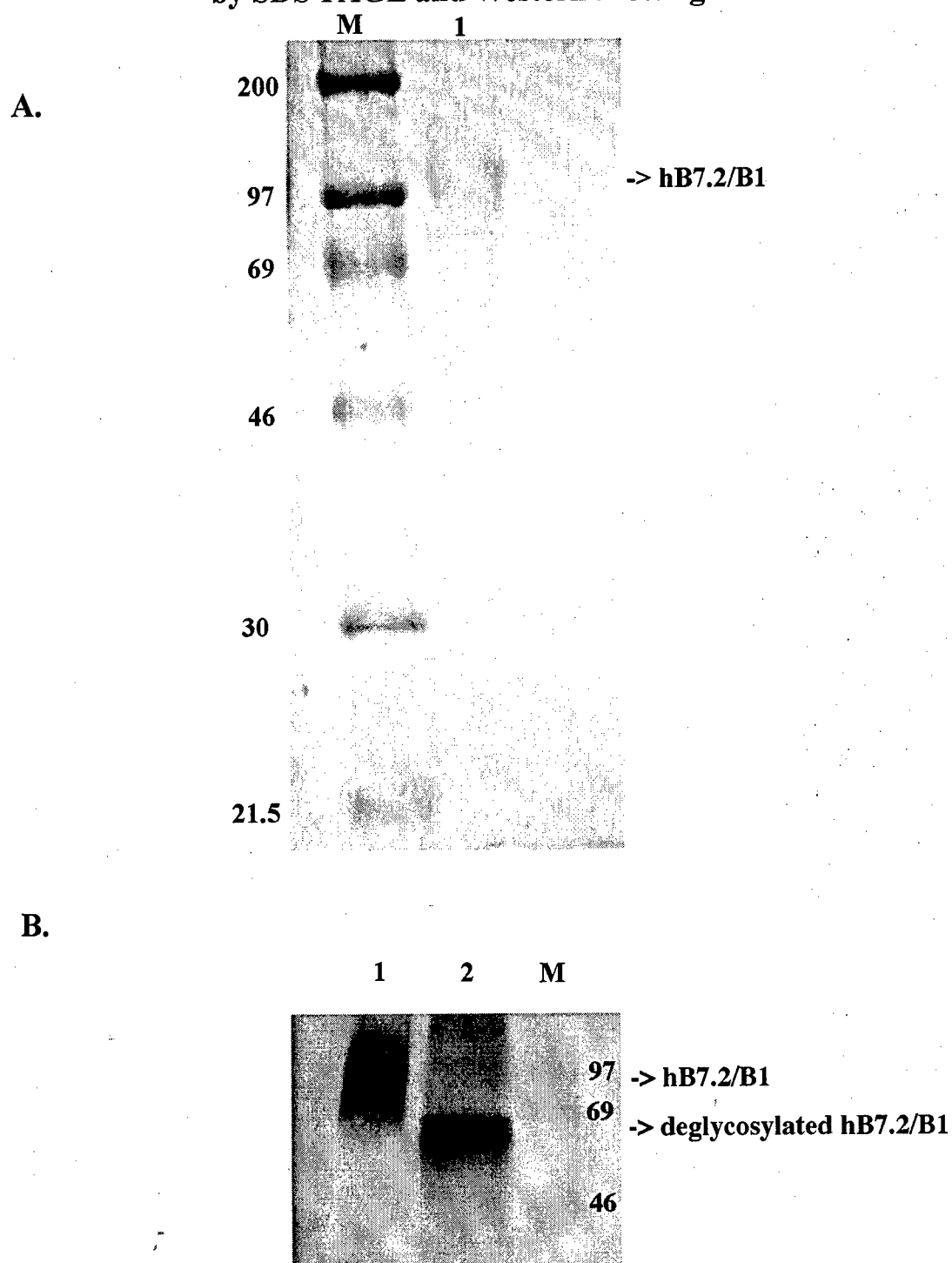97   -> hB7.2/B1

69   -> deglycosylated hB7.2/B1

46

FIGURE 3.5. Ability of HB7.2/B1 Fusion Protein to Bind to Counter Receptors as Determined by ELISA.

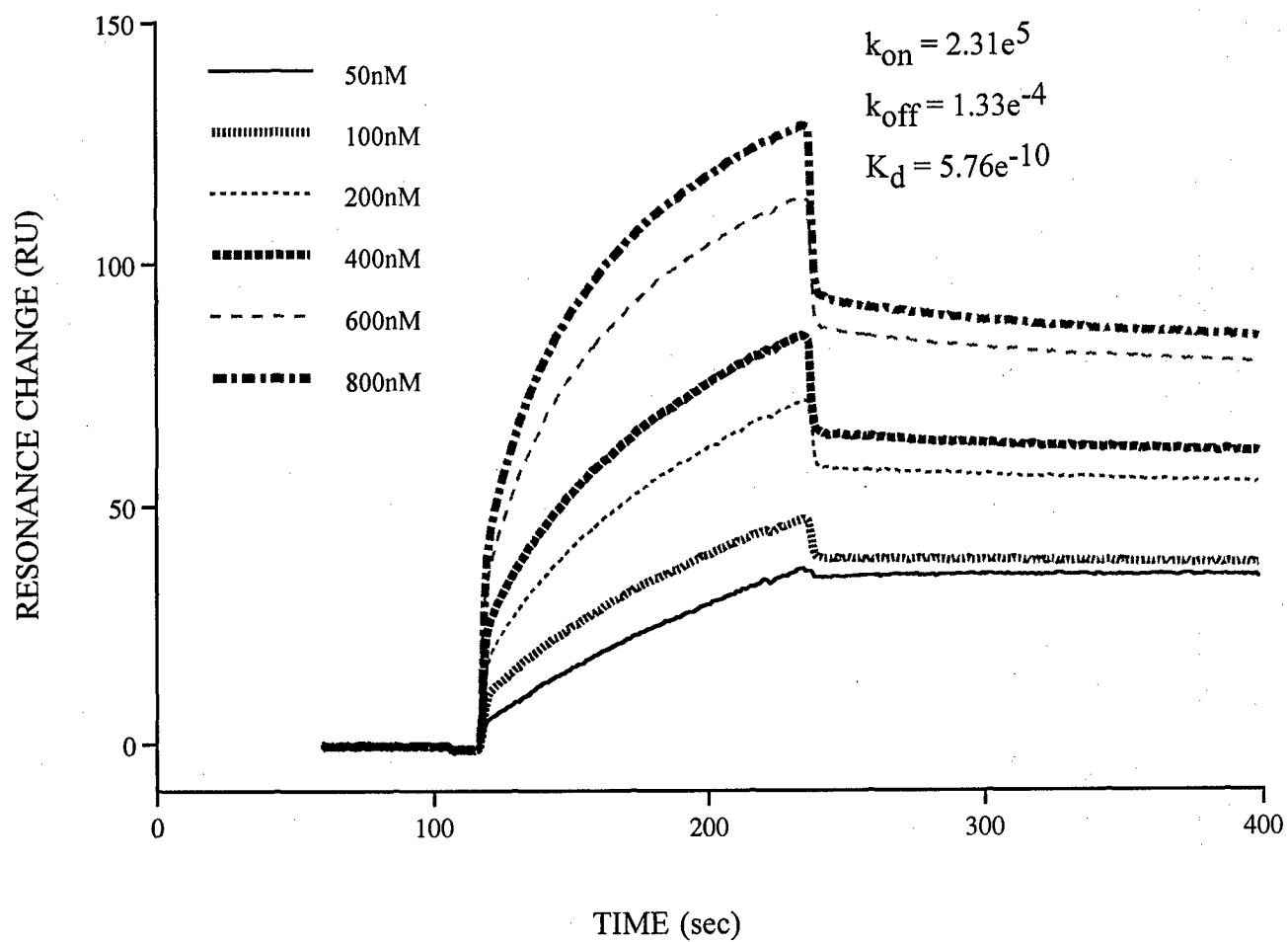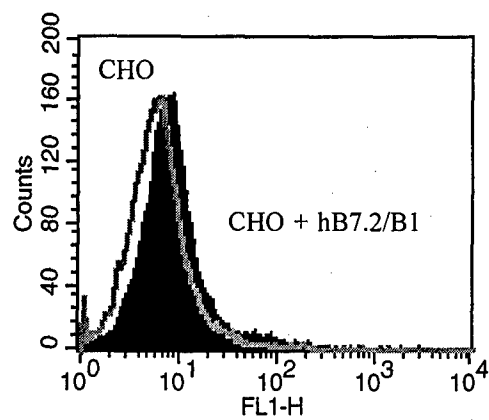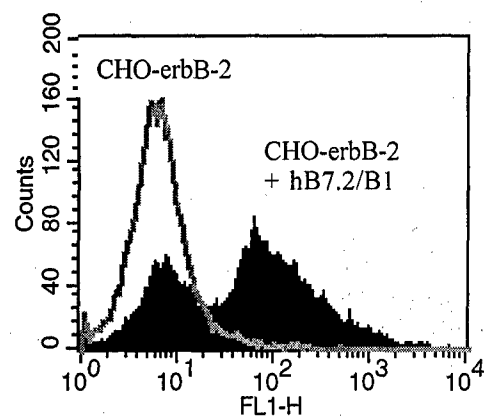# FIGURE 3.6 Kinetics of HB7.2/B1 as Measured by BIAcore Analysis

Legend:
- 50nM
- 100nM
- 200nM
- 400nM
- 600nM
- 800nM

$k_{on} = 2.31e^5$

$k_{off} = 1.33e^{-4}$

$K_d = 5.76e^{-10}$

Y-axis: RESONANCE CHANGE (RU)

X-axis: TIME (sec)
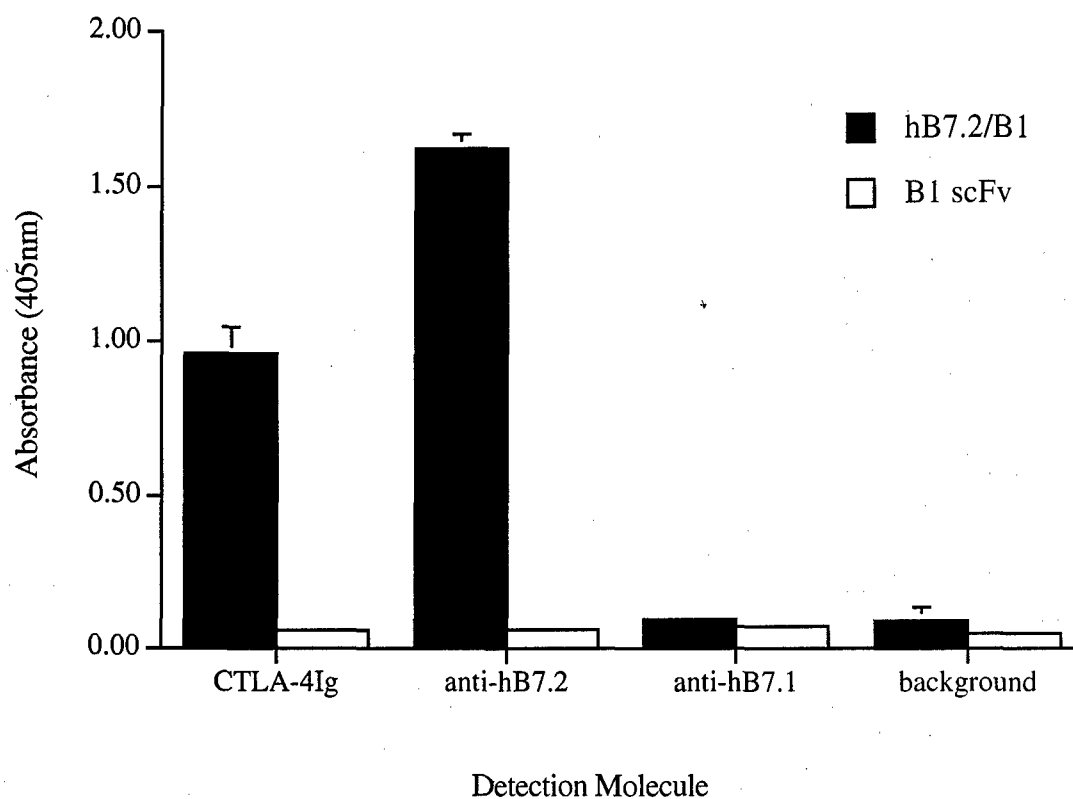
FIGURE 3.7. Cell Surface Binding of HB7.2/B1 Fusion Protein to Transfected CHO Cells

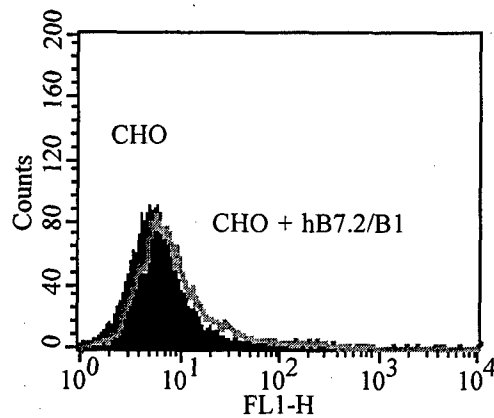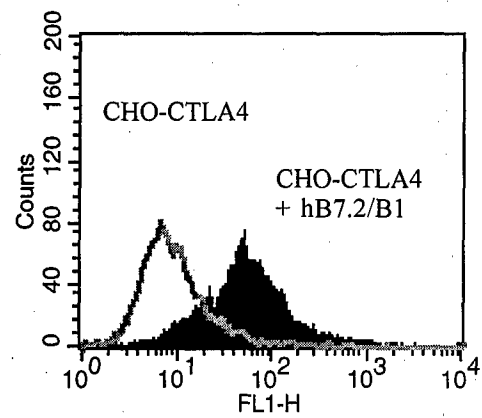FIGURE 3.8. T Cell Costimulation Utilizing Latex Beads Coated with HB7.2/B1.

[IL-2]ng/ml

Latex Beads and Inhibitor Added

no beads

anti-CD3 beads

anti-CD3 + anti-CD28 beads

anti-CD3 + hB7.2/B1 beads

anti-CD3 + hB7.2/B1 beads + anti-hB7.2 mAb

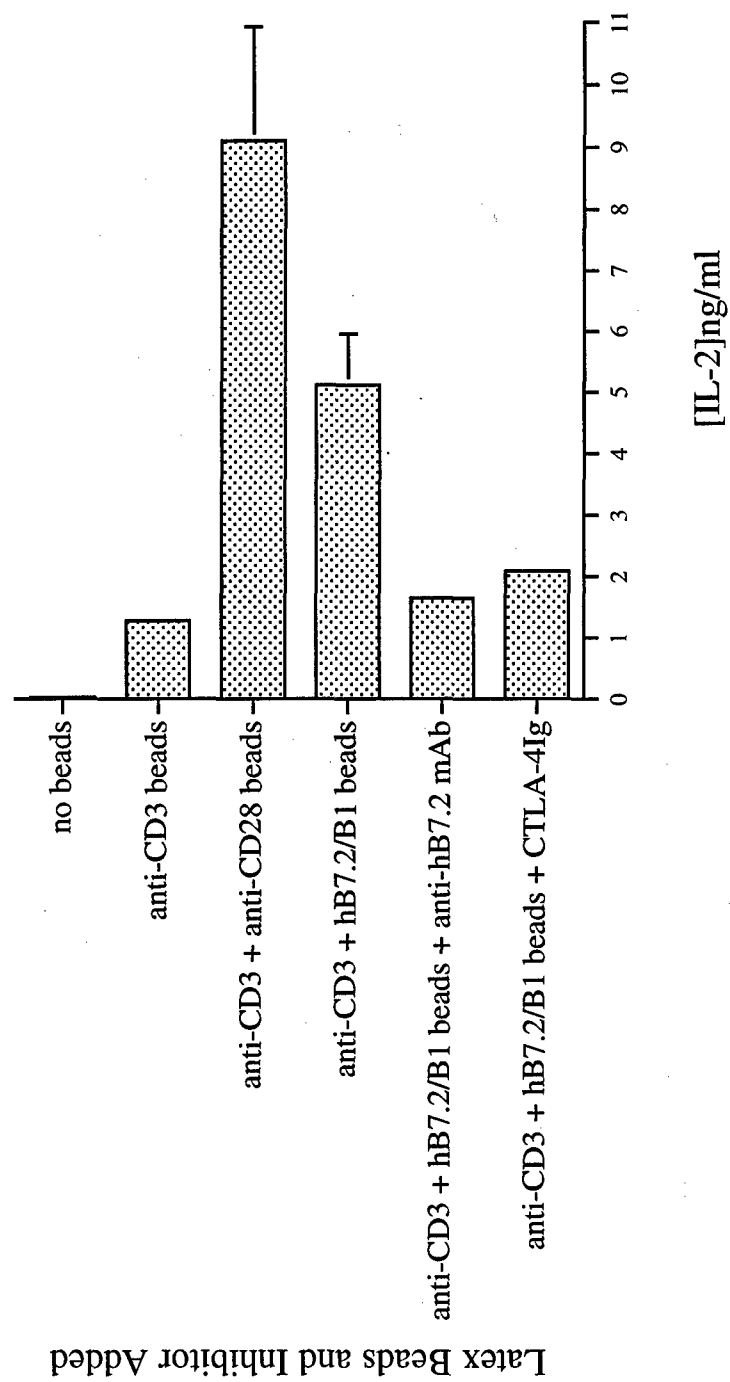anti-CD3 + hB7.2/B1 beads + CTLA-4Ig

FIGURE 3.9. T Cell Costimulation Utilizing CHO-erbB-2 Transfectants and HB7.2/B1.