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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 c-erbB-2 (Her2/neu), an oncogene product overexpressed by 30-50% of breast carcinomas, and the extracellular domain of B7-2 (CD86). 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. 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.

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K. B. M. L.L. August 24, 1998

PI - Signature Date

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1. Introduction

1.1. Breast Cancer and Current Strategies for Therapy

Breast cancer is the leading cause of cancer death in women. Current treatments include; i) the surgical removal of tumors or complete mastectomies; ii) radiation treatment; and iii) chemotherapy using radioisotopes, immunotoxins, or other drugs. None of these treatments are completely efficacious, and all have significant side effects (1, 2). The development of new therapeutics with significantly increased cure rates and dramatically reduced side effects is essential for the acceptable treatment of this disease.

1.2. Costimulation in Antigen Specific Immune Responses

Contact of the T cell receptor with MHC-peptide complexes is necessary but not sufficient for T-cell activation (3). If T cells receive the primary MHC-peptide signal, but a second, costimulatory signal is not received, then the T cells may enter a state of long-term unresponsiveness termed anergy, leaving them ineffective at eliminating the target cells (4, 5). The most significant receptor/ligand pair that can provide the costimulatory signal is thought to be the B7 molecule on antigen presenting cells (APC) interacting with CD28 on T cells (6, 7). After T cell activation, another B7 receptor is expressed on T cells and is thought to contribute to down-regulation of T cell responses (8). This second B7 receptor, termed CTLA-4, is expressed on T cells approximately 48 hours after activation and binds B7 molecules with a twenty fold higher affinity than CD28 (9). Thus, CTLA-4 may complete with CD28 for B7 binding, resulting in diminished CD28 T cell signaling (8).

Both B7 and CD28 are glycoprotein members of the Ig supergene family of cell surface molecules with each having a single IgV domain (reviewed in ref. (10)). CD28 is generally expressed as a homodimer whereas B7 is monomeric. Two different B7 molecules have been cloned to date and have been termed B7.1 (CD80) (11) and B7.2 (CD86) (12). B7.2 has been shown to be constitutively expressed, whereas B7.1 expression appears to be induced after B cell activation. The expression of B7 molecules is generally limited to APC, including macrophages, activated B cells, and dendritic cells. Human and murine CD28 are highly homologous at the amino acid level with 69% amino acid identity and, therefore, human B7 (hB7) can effectively signal murine CD28 (Dr. John Imboden, personal communication).

1.3. Costimulatory Signaling and Cancer Therapy

The fact 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 is 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 serve as APC in T cell activation. Indeed, recent experiments support the concept that provision of either B7.1 or B7.2 to tumor cells can result in their elimination in a cytotoxic T

lymphocyte (CTL) dependent manner (13 - 19). Several investigators have transfected murine tumor cells with either B7 alone, or in combination with MHC molecules, and determined their effects on tumor cell growth *in vivo* (13 - 19). In general, tumor cells transfected with B7 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 were also able to stimulate *in vitro* T cell proliferation and cytotoxic T cell responses (20). These experiments suggest that treatment with B7 transfected tumor cells could induce protective immunity by active immunization as a treatment for human cancers. Whether the B7 transfected tumor cells function solely by directly stimulating an anti-tumor CTL response or trigger the involvement of other immune cells is still unclear. However, these results suggest that provision of T cell costimulatory molecules to tumor cells will lead to tumor rejection.

1.4. Antibodies to the Breast Carcinoma Marker c-erbB-2

Specific delivery of molecules to tumor cells can be accomplished using antibody based targeting. A number of strategies have been devised for antibody based cancer therapy, including coupling tumor specific antibodies to immunotoxins, radioisotopes, and molecules that are involved in triggering an immune response. The underlying requirement of such strategies is the development of an anti-tumor antibody. Tumor specific antibody development 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 c-erbB-2, which has been demonstrated to be overexpressed in 30-50% of human breast carcinomas (21, 22).

Until recently, mouse IgG were primarily used for antibody based tumor targeting, resulting in a number of limitations. IgG are large (150kD) molecules which diffuse slowly into tumors (1 mm every 2 days) (23). The large size of IgG also results in slow clearance from the body and poor tumor:normal organ ratios (24). If the antibody carries a toxic agent, significant bystander damage may result. Recent advances in molecular biology have made it possible to produce $(Fab')_2$ and Fab in *E. coli*, as well as even smaller single chain Fv molecules (scFv, 25kDa). The scFv consist of the heavy and light chain variable regions (V_H and V_L) connected by a flexible peptide linker which retain the binding properties of the IgG from which they were derived (25). Smaller antibody molecules, particularly scFv, are cleared from the blood more rapidly than IgG, and thus provide significantly greater targeting specificity (26). scFv also penetrate tumors much better than IgG in preclinical models (27).

Another 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 (28). The smaller size antibody fragments should be less immunogenic, but this still may be a problem

when repeated doses are required for therapy. Thus therapeutic antibodies would ideally be of human origin. Unfortunately, production of human antibodies using hybridoma technology has proven extremely difficult, especially antibodies against human proteins such as tumor antigens (29).

All of the above limitations can be overcome by taking advantage of recent advances in biotechnology to produce human antibody fragments directly in bacteria without immunization (reviewed in 30, 31). Bacterial libraries containing billions of human antibody fragments are created, from which binding antibody fragments can be selected by antigen. Antibody fragments are entirely human in sequence, and thus less immunogenic than murine or humanized antibodies. In addition, affinity can be increased *in vitro* to < 1 nM (32).

The technology described above has been used to produce high affinity human scFv which bind to c-erbB-2. The initial isolate, C6.5, came from a non-immune human scFv phage antibody library (33), bound c-erbB-2 with a K_d of 1.6 x 10^{-8} M, and resulted in specific binding to c-erbB-2 expressing SK-OV-3 tumor xenografts in scid mice (34). At 24 hours, however, less than 1% of the injected dose (ID) was retained per gram (gm) of tumor (34). To increase retention, mutant phage antibody libraries were created by chain shuffling, or by mutating the antibody complementarity determining regions (CDRs). A C6.5 mutant (C6L1) with a 6 fold increased affinity for c-erbB-2 (K_d = 2.5 x 10^{-9} M) was created by chain shuffling (35). The 24 hour tumor retention of C6L1 was almost twice as great as for C6.5 (1.13% ID/gm vs. 0.67% ID/gm, $p<0.048$) with significantly higher tumor to normal organ ratios. Mutation of the C6.5 V_L CDR3 and V_H CDR3 resulted in a C6.5 mutant (C6MH3-B1) with a 100 fold increased affinity (K_d = 1.6 x 10^{-10} M)(36). C6MH3-B1 represents the highest affinity tumor targeting antibody produced by any means to date. In the process of producing C6MH3-B1, the necessary techniques to rapidly and efficiently create, select and characterize higher affinity phage antibodies has also been developed.

1.5. Bispecific Antibodies and Fusion Proteins Combining Anti-c-erbB-2 and Costimulatory Molecules

The high affinity human anti-c-erbB-2 scFv provide a means for delivering B7 to breast cancer cells for *in vivo* T cell activation. A single polypeptide chain fusion protein can be constructed consisting of anti-c-erbB-2 scFv and B7. The fusion protein would be entirely human in sequence, would bind to c-erbB-2 expressing breast tumor cells with high affinity, and should provide the costimulatory molecule for T cell activation. An alternative approach would be to provide costimulation by replacing the B7 portion of the fusion protein with a scFv directed against CD28. This could be accomplished by creating a bispecific antibody fragment consisting of anti-c-erbB-2 scFv and an anti-CD28 scFv .

Bispecific antibodies have been produced which trigger the immune system, however none attempt 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 (37, 38). A limitation of using CD3, however, is that crosslinking of CD3 by the bispecific antibodies results in T cell activation regardless of MHC expression, immunogenic peptide display, or T cell restriction.

Thus there are theoretical advantages to activating T cells via costimulatory molecules 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-c-erbB-2 antibody coupled to CD3, all cells expressing c-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 anti-c-erbB-2/B7 or anti-CD28 chimeras. In addition, the severe toxicity associated with the use of immunotoxins or radioisotope antibody conjugates would be avoided. Secondly, tumor cells need not express c-erbB-2 to be targeted. Provided that immunogenic peptides displayed by MHC molecules are common between c-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 c-erbB-2 marker (16). The importance of this point should not be understated; even tumor cells which lose expression of the target marker will 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.

1.6. Hypotheses

Hypothesis 1

Tumor antigen specific T cell activation can be achieved using a novel, bifunctional fusion protein which incorporates an anti-tumor antibody and the extracellular domain of the costimulatory molecule B7. The fusion protein should bind with high affinity to cancers and provide B7 for T cell costimulation, resulting in tumor specific T cell activation, proliferation, and tumor cell cytolysis.

Hypothesis 2

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 (39). Development of a number of anti-CD28 scFv with a wide range of affinities will be useful in exploring the relationship between affinity and T cell activation through the CD28 receptor. 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 can be used with anti-c-erbB-2 scFv to make bispecific antibodies for tumor therapy.

1.7. Recent Publications Describing B7/Anti-c-erbB-2 Fusion Proteins

In the last year, two reports have been published which describe the construction and characterization of fusion proteins combining an anti-c-erbB-2 antibody and either B7.1 or B7.2 (40, 41), thereby supporting the first hypothesis stated above. In both cases, the costimulatory molecule was engineered at the N-terminus of the fusion protein, an important point that will be discussed further below. Both groups showed that B7/anti-c-erbB-2 fusion proteins localizes to c-erbB-2 bearing cells and provides the costimulatory signal to T cells, resulting in increased T cell proliferation.

2. Body

2.1 Experimental Methods

Construction of the anti-c-erbB-2/B7 gene

To generate the anti-c-erbB-2/B7 fusion proteins, the genes for CD80(B7.1) and CD86(B7.2) were PCR amplified from vectors containing the genes (gift of Dr. Lewis Lanier, DNAX) with primers containing *ApaL1* or *Not1* restriction site overhangs and individually subcloned *ApaL1/Not1* into pHenIX vectors. Anti-c-erbB-2 scFv genes were subsequently subcloned *Sfi1/Xho1* into the pHenIX vectors already containing the B7 genes. Correct clones were identified by DNA sequencing.

Expression and purification of original fusion protein

The anti-c-erbB-2/B7 constructs were subsequently subcloned *Sfi1/Not1* from the pHenIX vectors into the mammalian expression vector pSecTag.B (Invitrogen) which contains the CMV promoter, Vk Ig leader sequence for protein secretion, and the *myc* epitope and $(His)_6$ tags for detection and purification, respectively. CHO cells were transfected by the calcium phosphate method and stable transfectants selected by growth in RPMI 1640 supplemented with 2mM glutamine, 10% FCS, and 1mg/ml G418. Fusion protein was expressed in CHO cells and purified by IMAC (42) exactly as described in (34).

Construction of the B7/anti-c-erbB-2 fusion protein in the opposite orientation

To generate the B7/anti-c-erbB-2 fusion protein in the opposite orientation, the gene CD86 (B7.2) was PCR amplified from a vector containing the gene (gift of Dr. Lewis Lanier, DNAX) with primers containing *Nco1* or *Xho1* restriction site overhangs and subcloned *Nco1 /Xho1* into pHenIX vectors. Anti-c-erbB-2 scFv genes C6.5, ML3-9, B1, and G98A were individually subsequently subcloned *ApaL1/Not1* into the pHenIX vectors already containing the B7 genes. Correct clones were identified by DNA sequencing.

Cloning into *Pichia* expression vectors

Correct clones were next subcloned into a *Pichia pastoris* expression vector, PiczαB (Invitrogen), using *Sfi1/Not1* cloning sites. The E. Coli strain TG1 was transformed with ligation products, screened for the presence of inserts, and used to

make large scale plasmid preps for Pichia transformation. Positive clones were sequenced before transforming Pichia strain GS115 by electroporation. Pichia transformants were screened by digestion with *Xho1/No1* to give two bands around 700 base pairs.

Expression and purification of recombinant proteins

Small scale expression was performed to screen for good expressors of each clone. In all cases, fusion protein 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 fusion proteins from culture, cellular debris was removed by centrifugation and supernatents were adjusted to pH8.0 and run over an IMAC column. Elute was concentrated and, if necessary, further purified by gel filtration over a Superdex 200 column. Purified protein was analyzed by SDS-PAGE and Western blotting using both 9E10 (anti-myc tag) or polyclonal anti-C6.5 rabbit serum for detection.

Binding assays

The ability of fusion proteins to simultaneously bind both c-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 c-erbB-2 (gift of Jim Huston, Creative Biomolecules) immobilized on 96 well plates (Nunc; Maxisorp). Fusion protein was allowed to bind and detected by addition of CTLA-4Ig (gift of Dr. Mark de Boer) or anti-B7 mAb (PharMingen), HRP conjugated anti-human Fc or anti-mouse mAbs, and incubation with peroxidase substrate followed by reading at 405nm by ELISA plate reader.

Cell surface binding assays

Cell surface binding of the fusion proteins are performed by FACS analysis on a FACScan (Becton Dickinson). Adherent cells are removed from culture flasks by ten minute incubation in cell dissociation buffer (Gibco), and washed two times in PBS. For binding to c-erbB-2, either cell lines CHO or CHO-erb transfectants (provided by Dr. J. Rosenblatt) are incubated at 5x10^5 with either 0.1 or 1.0ug of fusion protein for 45 minutes at 4°C. Cells are washed twice and stained by incubation with either 9E10 or anti-B7 mAb and FITC labelled goat anti-mouse IgG (Sigma) for 30 minutes. For CTLA-4 binding, CHO-CTLA-4 transfectants (provided by U. Pessara) were used and the combination of 9E10 and FITC labeled goat anti-mouse IgG utilized for detection.

Construction, expression, and purification of bispecific scFv

Bispecific scFv containing anti-CD28 scFv were made by subcloning the anti-CD28 scFv genes into the original pHenIX fusion protein vector, *ApaL1/Not1.* Bispecific scFv were expressed in *E. Coli* and purified by IMAC exactly as described above for the original anti-c-erbB-2/B7 fusion protein.

Determination of kinetics by surface plasmon resonance

Kinetics were measured by surface plasmon resonance on a BIAcore. Briefly, CD28Ig was immobilized on a CM5 sensor chip using NHS-EDC chemistry in 10mM Na-acetate pH 5.0. Associations were measured under continuous flow of 15ul/minute using a range of concentrations. 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 CD28Ig. K_d was calculated as k_{off}/k_{on}.

2.2 Assumptions

In an effort to develop a therapeutic capable of activating an anti-tumor immune response, the construction of two different chimeric molecules was proposed. If each half of the chimeric molecules is available for binding, and the orientation of the molecules is appropriate for simultaneous binding to c-erbB-2 on tumor cells and to CD28 on T cells, then these molecules should be able to induce activation and proliferation of T cells. I have proposed two different ways of engaging CD28 (either via B7 or an anti-CD28 antibody) to increase the likelihood of producing a molecule that is capable of simultaneous bindingboth receptors.

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. However, as will be discussed below, our group and others have found that the orientation is of considerable importance.

2.3 Procedures

Initial anti-c-erbB-2/B7 fusion protein

As described in last year's report, a chimeric fusion protein was engineered by genetic linkage of a scFv specific for the tumor antigen c-erbB-2 and the extra cellular domain of the T cell costimulatory molecule B7. The two fragments of the fusion protein genes are separated by a gene segment which encodes the 15 amino acid linker $(G_4S)_3$. This linker joined the C-terminus of the anti-c-erbB-2 scFv antibody fragment and the N-terminus of the B7 protein.

Initial binding experiments using surface plasmon resonance (SPR), in a BIAcore, showed that the anti-c-erbB-2/B7 fusion protein was bifunctional. When the fusion protein was allowed to flow over a c-erbB-2 ECD coated surface, a resonance unit (RU) change was observed as the fusion protein bound the immobilized tumor antigen. Subsequent addition of CTLA-4Ig resulted in a further increase in the RU bound.

Since last year's report, a number of experiments have been conducted to show that the fusion protein can bind to CD28 expressing cells without success. In addition, the fusion protein was unable to costimulate T cells in a T cell assay measuring IL-2 production.

In the publication by Rosenblatt and colleagues describing the construction and characterization of a similar fusion protein, it was noted that early attempts to

construct this fusion protein and show binding to CD28 failed when the B7 molecule was engineered into the C-terminus of the molecule (41). Apparently, the amino terminus of B7 molecules is required for binding to the T cell receptor and fusion to this end of the molecule prohibits their optimal binding.

Reconstruction of the fusion protein in the opposite orientation

Because both the Wels and Rosenblatt groups have had success with fusion proteins containing B7 at the N-terminus, and because our fusion protein was not effective in binding CD28 or in costimulating T cells, a new fusion protein was constructed in the opposite orientation (40, 41). The Wels group chose a *Pichia* expression system for their molecule that has been recently utilized in the Marks' lab for scFv fusion proteins. We thus chose to remake the fusion protein in the opposite orientation and produce the fusion protein in *Pichia pastoris*.

The gene for B7.2 and one of four different anti-c-erbB-2 scFv genes, C6.5, ML3.9, B1, and G98A were subcloned into the pHenIX vector, sequenced for correctness, and subsequently subcloned into the Pichia vector, PiczαB. Large scale plasmid preps of PiczαB containing the fusion protein were prepared and used to transform the *Pichia* strain GS115. Expression of the fusion protein in PiczαB is controlled under the methanol-inducible alcohol oxidase 1 promoter and the vector contains the α factor secretion signal for yeast and both the myc and $(His)_6$ tags at the C-terminus.

Expression, purification, and characterization of the fusion protein constructed in the opposite orientation

Initial expression of the re-engineered fusion protein was performed in shaker flasks and purified by IMAC. Analysis by SDS-PAGE and western blotting confirms that the fusion protein is purified to >90% and runs as a wide band between 80-100kDa instead of the calculated 57kDa due to glycosylation (data not shown). The best expresser thus far is the B7/B1 fusion protein. The anti-c-erbB-2 scFv, B1(C6MH3-B1), has the greatest affinity for c-erbB-2 among the four anti-c-erbB-2 scFv, with a sub-nanomolar apparent affinity ($K_d = 1.6 \times 10^{-10}$ M).

B7/B1 appears bifunctional by ELISA using c-erbB-2 ECD coated plates and detecting with either anti-B7.2 or CTLA4Ig followed by an HRP conjugated antibody (**Figure 1**). To test whether the fusion protein can bind to the target molecules on the cell surface as compared to immobilized recombinant material, cell staining experiments were performed and binding measured by fluorescence cytometry. Two different transfected CHO cell lines were utilized for these experiments. The c-erbB-2 transfected cell line, CHO-erb, was used to show that the anti-c-erbB-2 half of the fusion protein can bind to c-erbB-2 on the cell surface (Figure 2). Likewise, the CTLA-4 transfected cell line, CHO-CTLA4, was used to test whether the B7 component is functional on cells (Figure 3). In both cases, the two components of the B7/B1 fusion protein appear capable of binding to cells through the appropriate receptors. In contrast to the original fusion protein, the re-engineered molecule showed more favorable binding properties and, thus, should more likely work as envisioned to provide T cells with the costimulatory signal for optimal activation.

We are now working toward testing this newly constructed fusion molecule in T cell activation assays.

Affinity and Kinetics of anti-CD28 scFv

In last year's report, we described the selection of several different anti-CD28 scFv isolated by screening phage displaying human antibody fragments on immobilized CD28Ig. After four rounds of selection utilizing human CD28Ig and human IgG1, five putative scFv were isolated which appear to be specific for the CD28 portion of chimeric CD28Ig. Characterization of these clones was to be performed by DNA sequence analysis, binding kinetics on CD28Ig, and T cell costimulation assays.

Since then, large scale expressions were performed on the five different scFv clones identified. To determine the affinity and kinetics of these anti-CD28 scFv, each clone was purified by IMAC, gel-filtered to separate monomer from higher molecular weight aggregates, which display greater avidity, and immediately tested for binding to immobilized CD28Ig on a BIAcore (Figure 3). The affinities of each scFv clone for CD28Ig were determined, as was each association and dissociation rate (Table 1). One of the scFv clones showed negligible binding to CD28Ig and was not tested further.

The clones showed a range of affinities for CD28Ig from 130 to 600nM, considerably better than that of B7 for CD28 (4uM). One of these scFv also binds CTLA-4Ig with an apparent affinity of 7uM. The other scFv show negligible binding to CTLA-4Ig.

To date, no other group has reported generating scFv specific for CD28. Bispecifics incorporating an anti-c-erbB-2 scFv and also a scFv that is specific for CD28 may be superior to fusion proteins using B7 because negative signaling through CTLA-4 would be eliminated.

Thus far, experiments attempting to show cell surface binding of CD28 by these anti-CD28 scFv have been unsuccessful. Because scFv lack the avidity of whole antibodies, they need very slow off rates to be detected in cell surface binding experiments as measured by FACS. Other possible reasons for the lack of detectable binding include, 1) cell surface CD28 is conformational different that immobilized CD28Ig used for selections and for BIAcore studies, and 2) the Ig portion of CD28Ig may make up an overlapping part of the anti-CD28 scFv epitope. Further studies on the anti-CD28 scFv are currently on hold.

2.4 Results and Discussion

Fusion Proteins

Because our group and others have found that fusion proteins incorporating B7 at the C-terminus have lower affinity for receptor binding, the B7/anti-c-erbB-2 fusion protein was reconstructed in the opposite orientation. The re-engineered fusion molecule links the C-terminus of the B7 protein and the N-terminus of the anti-c-erbB-2 scFv antibody fragment. Initial experiments with one of the newly

constructed fusion proteins, B7/B1, showed cell surface binding both to c-erbB-2 and to CTLA-4. T cell activation assays are currently being readied.

Anti-CD28 scFv antibodies

By using a human scFv phage library and selecting on CD28Ig, four putative single chain antibody fragments have been isolated. The clones showed a range of affinities for CD28Ig from 130 to 600nM, considerably better than that of B7 for CD28 (4uM). One of these scFv also binds CTLA-4Ig with an apparent affinity of 7uM. The other scFv show negligible binding to CTLA-4Ig.

To date, no other group has reported generating scFv specific for CD28. Bispecifics incorporating an anti-c-erbB-2 scFv and also a scFv that is specific for CD28 may be superior to fusion proteins using B7 because negative signaling through CTLA-4 would be eliminated.

So far, attempts to show cell surface binding of these anti-CD28 scFv have been unsuccessful. A number of different strategies have been utilized without success. At this point, further studies on the anti-CD28 scFv are on hold while experiments are performed to show that the B7/anti-c-erbB-2 fusion protein can enhance T cell activation.

2.5 Recommendations to Statement of Work

Original Statement of Work (from proposal)

Specific Aim 1: Engineer a fusion protein incorporating anti-c-erbB-2 scFv and B7-2.

Task 1: Months 1-4: Genetically engineer fusion protein construct and confirm it is correct by DNA sequencing.

Task 2: Months 4-6 Express fusion protein and purify adequate quantities for in vitro evaluation.

Specific Aim 2: Engineer a bispecific antibody incorporating anti-c-erbB-2 and anti-CD28 scFv.

Task 3: Months 1-6: Isolate scFv antibody fragments which bind CD28 by selecting a nonimmune scFv phage antibody library on immobilized CD28.

Task 4: Months 10-18: If necessary, increase the affinity of scFv with the desired binding characteristics by creating mutant scFv phage antibody libraries and selecting on immobilized CD28.

Task 5: Months 21-24: Engineer bispecific antibody by genetic or chemical methods.

Specific Aim 3: Characterize and test the fusion protein and bispecific antibody.

Task 6: Months 4-36: Characterize anti-CD28 scFv, fusion proteins, and bispecific antibodies with respect to DNA sequence, affinity, kinetics of binding, and T cell activation.

At 24 months into the project, we are currently on track in producing B7/anti-c-erbB-2 fusion proteins and in the testing of these molecules. It is anticipated that complete testing of two to four fusion proteins with different anti-c-erbB-2 scFv, by both cell surface binding experiments and T cell activation experiments, will be completed within the next six months.

Significant time was spent constructing and testing the original fusion proteins, that apparently were in an improper orientation, and on the anti-CD28 scFv which did not show cell surface CD28 binding. Further studies on the anti-CD28 scFv and bispecifics incorporating these anti-CD28 scFv are currently being contemplated. However, success with the re-engineered fusion proteins in T cell activation is now the primary priority.

3. Conclusions

- The original chimeric fusion protein linking the C-terminus of the anti-c-erbB-2 scFv antibody fragment and the N-terminus of the B7 protein was found to be in an orientation that was inappropriate for the proposed strategy.

- The fusion protein has been reengineered in the opposite orientation that is specific both for the tumor antigen c-erbB-2 and for the T cell costimulatory receptor CD28. Initial binding experiments demonstrate B7/B1 is bifunctional in that it can bind to both c-erbB-2 and CTLA-4Ig simultaneously. B7/B1 is also capable of binding both to c-erbB-2 and to CTLA-4 on the cell surface as detected by FACS analysis. Experiments are currently underway to determine if B7/B1 can bind to cell surface CTLA-4 and to CD28. T cell activation assays are currently being readied.

- By using a human scFv phage library and selecting on CD28Ig, five putative single chain antibody fragments have been isolated. Although these scFv appear to be functional in binding immobilized CD28Ig by ELISA and BIAcore, detectable binding to cell surface CD28 has yet to be measured.

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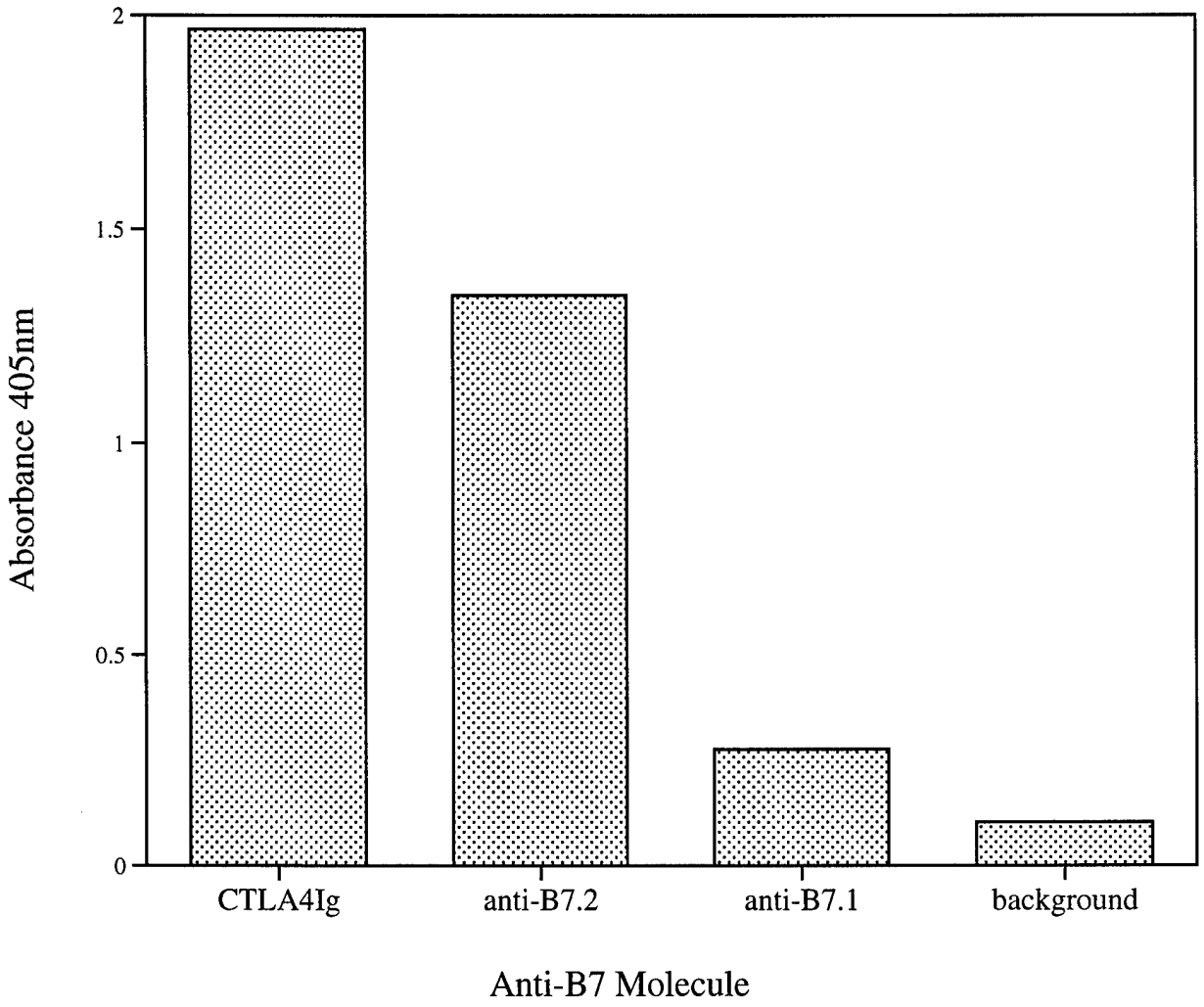

Figure 1. Comparison of different molecules used to detect the B7/B1 fusion protein in an ELISA. B7B1 was incubated on c-erbB-2 ECD coated plates and detected by addition of various anti-B7 molecules and HRP conjugated secondary antibody.

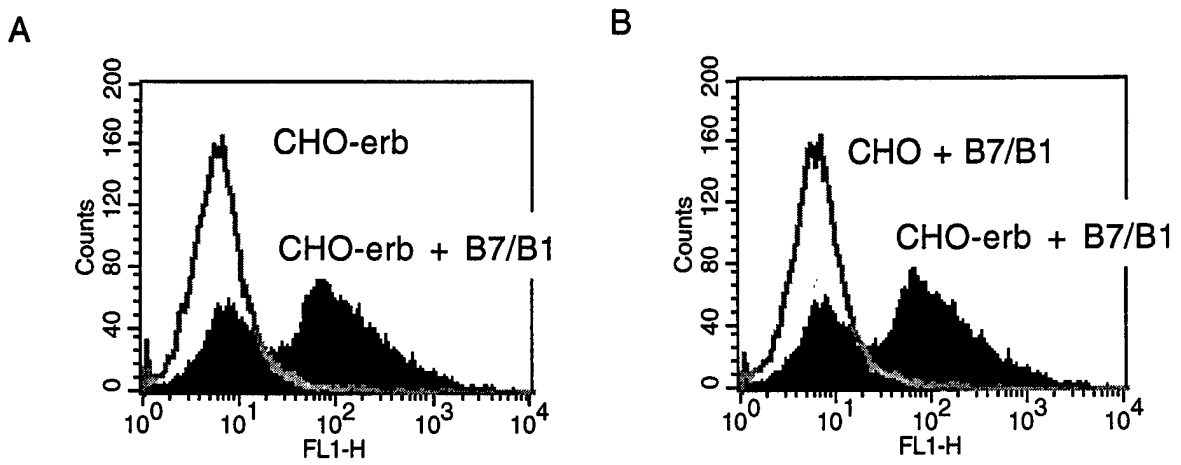

Figure 2. Binding of B7/B1 fusion protein to c-erbB-2 on a CHO-erb transfectant cell line as measured by FACS. B7/B1 was incubated at 1ug with 5x10^5 CHO cells or CHO-erb transfectants and binding detected with anti-B7 mAb and FITC labeled anti-mouse mAb. Both CHO-erb incubated with or without B7/B1 is compared (A) as is incubation of B7/B1 with either CHO cells or CHO-erb transfectants (B).

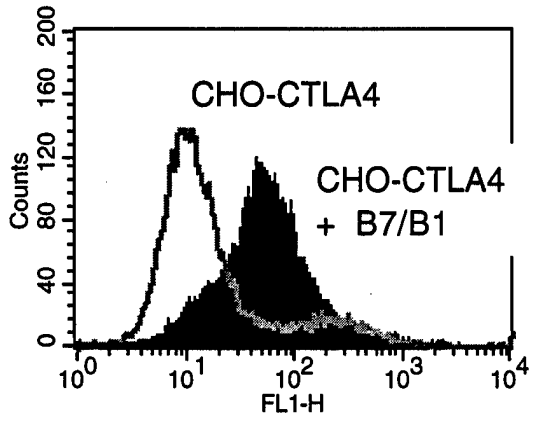

Figure 3. Binding of B7/B1 to cell surface CTLA-4 on CHO or CTLA-4 transfected CHO cell lines. B7/B1 was incubated at 1ug with 5x10^5 CHO cells or CHO-CTLA4 transfectants and binding detected with anti-myc tag 9E10 and FITC labeled anti-mouse mAb.

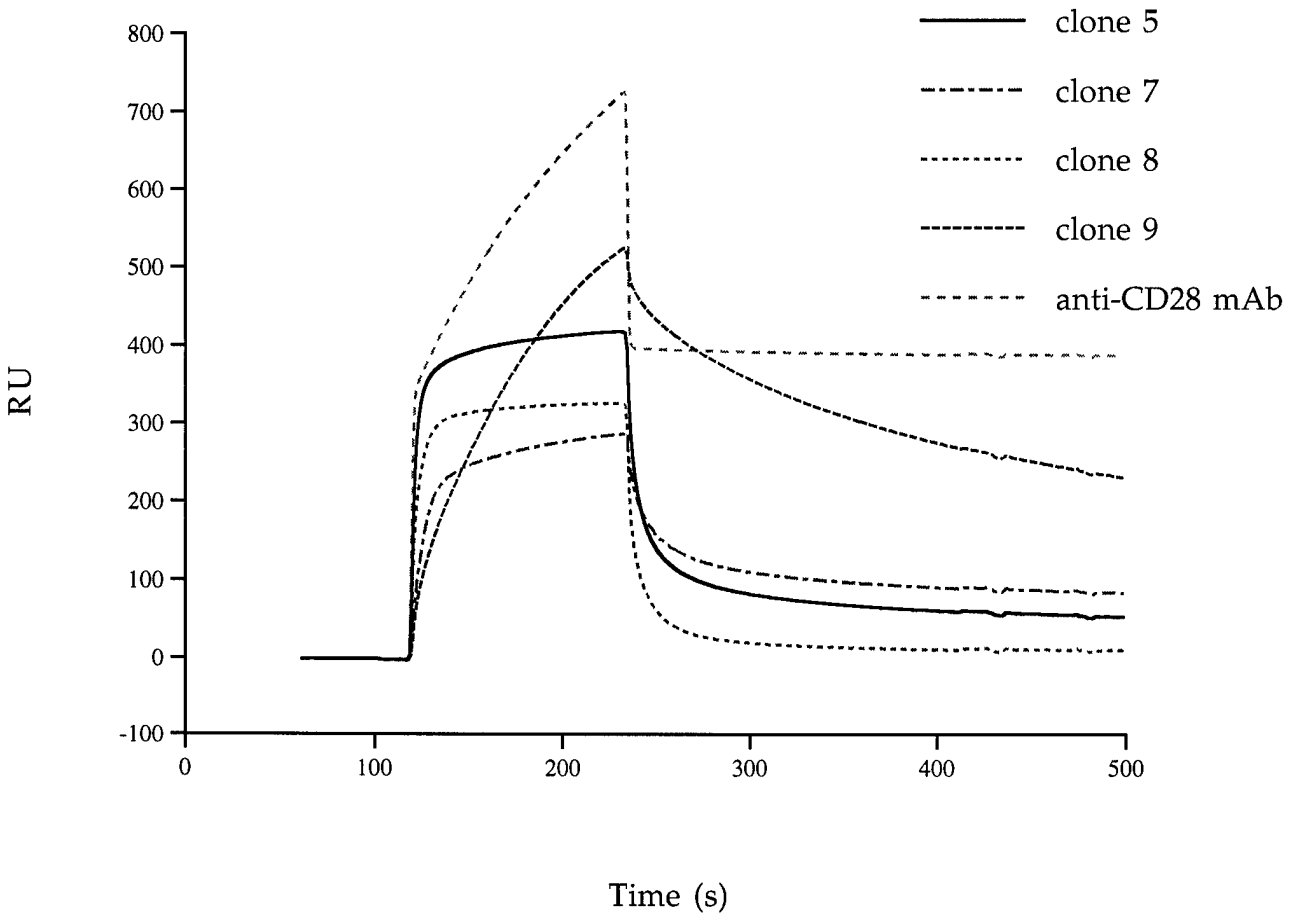

Figure 4. Overlay plot of anti-CD28 scFv binding to CD28Ig by BIAcore analysis. A BIAcore sensorgram demonstrating the ability of the anti-CD28scFv to bind CD28Ig. Immediately following gel filtration, scFv monomer were injected over a CD28Ig coated sensor chip and binding observed. k_{off}values were determined from this plot during the first two minutes of dissociation.

scFv clone	K_d (M)	k_{on} ($\times 10^4\ M^{-1}\ s^{-1}$)	k_{off} ($\times 10^{-3}\ s^{-1}$)	VH	VL	Shares epitope w/ anti-CD28 Mab	Also binds CTLA-4Ig
5	4.2×10^{-7}	13	54	DP.47/V3.2	DPL16/IGLV	yes	no
7	1.3×10^{-7}	22	28	DP.47/V3.2	DPK24/VkIV	no	no
8	6.0×10^{-7}	8.9	50	DP.47/V3.2	L12a	no	yes
9	nd	nd	nd	2M27/11M27	DPK4/A20	no	no

nd = not done

Table 1. Affinities, binding kinetics, andVH/VL gene usage of anti-CD28 scFv. Kinetics were measured by surface plasmon resonance on a BIAcore. Briefly, CD28Ig was immobilized on a CM5 sensor chip using NHS-EDC chemistry in 10mM Na-acetate pH 5.0. Associations were measured under continuous flow of 15ul/minute using a range of concentrations. 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 CD28Ig. K_d was calculated as k_{off}/k_{on}. The epitope studies were performed by coinjecting individual anti-CD28 scFv with anti-CD28. If the molecules share an epitope, then they will compete for binding to CD28Ig and the total RU bound will be less than should the molecules bind different epitopes.