Antibody-targeted interleukin 2 stimulates T-cell killing of autologous tumor cells

(recombinant antibody/cytokine fusion protein/tumor-infiltrating lymphocytes)

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ABSTRACT A genetically engineered fusion protein consisting of a chimeric anti-ganglioside GD2 antibody (ch14.18) and interleukin 2 (IL2) was tested for its ability to enhance the killing of autologous GD2-expressing melanoma target cells by a tumor-infiltrating lymphocyte line (660 TIL). The fusion of IL2 to the carboxyl terminus of the immunoglobulin heavy chain did not reduce IL2 activity as measured in a standard proliferation assay using either mouse or human T-cell lines. Antigen-binding activity was greater than that of the native chimeric antibody. The ability of resting 660 TIL cells to kill their autologous GD2-positive target cells was enhanced if the target cells were first coated with the fusion protein. This stimulation of killing was greater than that of uncoated cells in the presence of equivalent or higher concentrations of free IL2. Such antibody–cytokine fusion proteins may prove useful in targeting the biological effect of IL2 and other cytokines to tumor cells and in this way stimulate their immune destruction.

Much attention has been focused on the use of interleukin 2 (IL2) for cancer immunotherapy because of its stimulatory effect on a broad range of immune cell types, including both T and B cells, monocytes, macrophages, and natural killer cells. One class of cells resulting from in vitro or in vivo stimulation of immune cells has been called lymphokine-activated killer cells (1), and therapeutic approaches using such populations have shown clinical responses with some tumor types (2). Other, less refractory tumors may show greater responses if monoclonal antibodies directed against these tumors are used in combination with IL2 (3, 4). Such antibodies mediate antibody-dependent cellular cytotoxicity (ADCC) through their interactions with both the tumor cell antigen and the Fc receptor (CD16) present on certain subsets of natural killer cells, monocytes, granulocytes, and macrophages.

While IL2 treatment in vivo leads to increases in both natural killer and ADCC activities, the cytolytic activity of antigen-specific, major histocompatibility complex (MHC)-restricted T cells may actually be reduced (5). Treatment of T cells with anti-CD3 antibody prior to IL2 exposure greatly increases T-cell cytolytic activity (6). Likewise, expansion of tumor-infiltrating lymphocytes (TIL) by culture in the presence of high concentrations of IL2 with periodic target-cell stimulation leads to substantial increases in cytolytic activity (7). Both approaches involve costimulation of IL2 and T-cell antigen receptors for expansion and maintenance of T-cell cytolytic activity. Thus, an optimal therapy might combine IL2 activation and tumor antigen presentation together with a tumor-specific antibody that mediates both complement-dependent cytotoxicity (CDC) and ADCC activities. By combining a chimeric anti-ganglioside GD2 antibody (ch14.18) which has potent CDC and ADCC activities (8), with IL2, we hope to target this cytokine to tumors such as neuroblastoma (9, 10) and melanoma (11) expressing GD2. In this way, relatively large amounts of tumor antigens should be present during IL2 activation for expansion of cytotoxic T cells, since melanoma cell lines have been reported to express an average of 1.5 × 10^5 sites per cell for ch14.18 (8). Furthermore, the antibody would also be available to target Fc receptor-bearing cells that have been activated by the targeted IL2.

The ch14.18 antibody used in this report has already been shown to mediate potent ADCC activity by IL2-activated peripheral blood mononuclear cells from cancer patients (12). We have focused on the ability of a ch14.18–IL2 fusion protein to stimulate the proliferation and cytolytic activity of a human T-cell line against autologous melanoma targets. This cell line, 660 TIL, is CD3ε+, CD8ε+, antigen-specific, and MHC class I-restricted and was originally obtained by outgrowth from a human metastatic melanoma (13). A melanoma line, 660 mel, was derived from the same tumor and serves as a source for antigen stimulation and as an autologous target for 660 TIL (14). Results of this study show that tumor cells coated with a fusion protein in which IL2 is at the carboxyl terminus of the heavy-chain constant region 3 (CH3) exon of ch14.18 (CH3–IL2) efficiently stimulate resting 660 TIL cells to kill autologous targets. These coated cells serve as a model for tumors that have been targeted in vivo.

MATERIALS AND METHODS

Plasmid Constructs. The immunoglobulin–IL2 fusion protein expression vector was constructed by fusing a synthetic human IL2 sequence to the carboxyl end of the human Cε1 gene. A synthetic DNA linker, extending from the Sma I site near the end of the antibody coding sequence to the single PvuII site in the IL2 sequence, was used to join the amino-terminal codon of mature IL2 to the exact end of the CH3 exon (CH3–IL2). The fused gene was inserted into the vector pdHL2-14.18 as described earlier for an antibody–lymphotoxin fusion protein construct (15). Additional constructs were made in which the IL2 sequence was fused to the Sac I site in the hinge region of the human Cε3 gene (Fab–IL2) or to the end of the CH2 exon at a Taq I site (CH2–IL2). In both cases synthetic linkers were used to fuse the antibody and IL2 sequences directly without introducing any additional amino acid residues.

Transfection and Purification. The expression plasmids were introduced into Sp2/0-Ag14 cells by protoplast fusion and selected in Dulbecco’s modified Eagle’s medium (GIBCO) containing 10% fetal bovine serum and 100 nM

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; IL2, interleukin 2; MHC, major histocompatibility complex; TIL, tumor-infiltrating lymphocyte(s); C, constant; CH, heavy-chain C region; V, variable.
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methotrexate. Clones secreting the fusion proteins were identified by ELISA (16). The highest producers were grown in increasing concentrations of methotrexate and subcloned in medium containing 5 μM methotrexate. The CH3–IL2 fusion protein was purified using protein A-Sepharose (Repligen, Cambridge, MA) as affinity adsorbent. Small amounts of the Fab–IL2 and CH2–IL2 proteins were purified using an anti-human κ chain immunoaffinity column.

Antigen-Binding Activity. The antigen-binding activity was measured in a solid-phase ELISA using a chloroform extract of human neuroblastoma cells as a source of GD2 (17). In some cases the fusion proteins were first treated with plasmin (0.125 casein unit/ml) in 50 mM Tris, pH 8/150 mM NaCl for 1–2 hours at 37°C. Aprotinin (Sigma) was added at the end of the digestion (200 kallikrein inhibitory units/ml) when the digested protein was tested for antigen binding or IL2 activity.

Human TIL Culture. The 660 TIL line and its autologous GD2+ tumor line 660 mel were established from a human melanoma tumor sample and maintained in culture as described (14). IL2 Assays. IL2 activity of antibody–IL2 fusion proteins was assayed in standard T-cell proliferation assays using either the mouse CTLL-2 line (18) or 660 TIL. After IL2 depletion for 48 hr, 3 × 105 CTLL-2 cells or 105 660 TIL cells were added to individual wells of a 96-well microtiter plate in a volume of 0.2 ml with various concentrations of fusion protein (normalized for IL2 content) or recombinant IL2 [either yeast-derived (Genzyme) or bacteria-derived (Hoffmann-La Roche)]. After 72 hr, 0.5 μCi (18.5 kBq) of [methyl-3H]thymidine was added to each well, and plates were harvested 12 hr later. All samples were tested in duplicate.

Cytotoxicity Assays. Cytolytic activity of 660 TIL was measured in 31Cr-release assays against 660 mel target cells. The 660 TIL cells were depleted of IL2 for 4 days prior to their use in assays except where noted. Target cells (3 × 10⁴) were labeled with 300 μCi of Na35 CrO4 (Amersham) for 1 hr at 37°C and washed in RPMI 1640 with 10% heat-inactivated fetal bovine serum. For experiments in which target cells were coated with antibody, 35Cr-labeled target cells (10⁴ in 1 ml) were incubated with ch14.18 or CH3–IL2 fusion protein (50 μg/ml in RPMI 1640 with 10% heat-inactivated fetal bovine serum). After 1 hr at 4°C with periodic mixing, cells were washed three times with serum-containing medium to remove excess antibody and were used in cytotoxicity assays. In some experiments we evaluated the effect of adding either antibody or IL2 at the time of assay was determined. Duplicate assay mixtures were incubated at 37°C for 7–16 hr.

RESULTS

Characterization of Immunoglobulin–IL2 Fusion Proteins. Several forms of antibody–IL2 fusion proteins were constructed and expressed in transfected hybridoma cells. In initial studies we compared antigen-binding and IL2 activities of constructs consisting of a chimeric light chain, expressed in the same transfected cell with various truncated heavy chain–IL2 fusion proteins. In one case IL2 was fused to the beginning of the first hinge domain of the human C3 gene (deleting CH2 and CH3 exons) and in another construct IL2 was fused to the end of the CH2 exon (deleting CH3; Fig. 1A). These heavy-chain fusion protein constructs were expressed together with the variable (V) regions of the anti-GD2 antibody 14.18 and the human C3 gene. Secreted heavy chains were found to associate with the chimeric light chain to form Fab–IL2 or CH2–IL2 fusion proteins, but the latter did not assemble into a whole antibody even though it contained an intact hinge region (Fig. 1B). The covalent disulfide bonds that are normally involved in inter-heavy-chain binding are contained in the hinge.

![Table](https://example.com/table.png)

Table 1. IL2 activity of immunoglobulin–IL2 fusion proteins

<table>
<thead>
<tr>
<th>Construct</th>
<th>V region</th>
<th>Antigen binding</th>
<th>IL2 activity</th>
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<tbody>
<tr>
<td>Fab–IL2</td>
<td>14.18</td>
<td>–</td>
<td>5.0 × 10⁶</td>
</tr>
<tr>
<td>Fab–IL2</td>
<td>B72.3</td>
<td>+</td>
<td>6.0 × 10⁶</td>
</tr>
<tr>
<td>CH2–IL2</td>
<td>14.18</td>
<td>+</td>
<td>6.5 × 10⁵</td>
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IL2 activity in culture supernatants (without methotrexate) was determined by thymidine incorporation into mouse CTLL-2 cells. The amount of each fusion protein was determined by ELISA and the activity is reported as units/mg of IL2. Antigen binding was determined using GD2-coated (14.18) or mucin-coated (B72.3) plates as described (13).
The untreated CH3–IL2 protein had much higher binding activity in a direct antigen-binding assay than the chimeric antibody, and this enhanced activity was lost when IL2 was cleaved from the heavy chain with plasmin (Fig. 2). The gel analyses showed that the antibody itself was resistant to plasmin cleavage and that a heavy chain of the expected size was generated by cleavage at the antibody/IL2 junction. These results suggest that the fused IL2 domain actively interacts in some way with the antibody to alter antigen-binding activity. Upon removal of IL2, the normal level of activity is restored.

Since the CH3–IL2 construct was fully assembled into an antibody fusion protein, it is likely that this molecule has the most favorable properties for both in vitro and in vivo studies. It could also be readily purified by affinity chromatography on protein A-Sepharose. The availability of a matched set of TIL and its autologous tumor cell line, expressing the GD2 antigen, has allowed us to exploit this system as a model for testing the biological properties of antibody-targeted IL2.

Such a system was not available for a tumor cell expressing the TAG 72 antigen. For these reasons we have focused our studies on the characterization of the ch14.18 CH3–IL2 fusion protein.

**Biological Activities of Whole Antibody–IL2 Fusion Proteins.** The IL2 activity of the CH3–IL2 (14.18) fusion protein was tested in a standard T-cell proliferation assay using either the mouse CTLL–2 line or a human TIL line (660 TIL) established from a metastatic melanoma. Both lines were cultured without IL2 for 48 hr prior to assay. The activity of the fusion protein was found to be somewhat less than that of a recombinant IL2 made in bacteria but was identical to that of a recombinant IL2 preparation produced in yeast (2.5 × 10⁶ units/mg) when either the murine or the human T cells were used (Fig. 3). Thus, fusion of this cytokine to the carboxyl terminus of an antibody or antibody fragment does not significantly reduce its activity. We reported a similar result when we found that lymphotuxin (tumor necrosis factor β) retained its full activity when fused to the end of the CH3 domain (15). However, in that case some inactivation of lymphotuxin activity occurred during the elution from the protein A-Sepharose column. In contrast, the CH3–IL2 preparation used in this study was stable throughout the purification and during subsequent storage for up to 18 months at −20°C. The effector functions of the CH3–IL2 protein—i.e., the ability to mediate complement and Fc receptor-dependent lysis—were also tested and found to be maintained (but somewhat decreased) when compared with that of the chimeric 14.18 antibody (data not shown). A similar result was reported for the CH3–lymphotoxin fusion protein (15).

**Enhanced TIL Cytotoxic Activity of Autologous Tumor Targets.** The human 660 TIL line was used to test the ability of the CH3–IL2 (14.18) fusion protein to stimulate the killing of GD2+ autologous melanoma tumor cells (660 mel). The 660 TIL line is routinely cultured in serum-free medium containing IL2 (1000 units/mL) and is stimulated bimonthly with 660 mel to maintain killing activity (13). The lytic activity of this CD8+ cell line for its target varies over time in culture as a function of antigen stimulation. For the purpose of this study we have also examined the effect of IL2 depletion on TIL cytotoxic activity and how this might be affected by subsequent addition of IL2 or the CH3–IL2 fusion protein. Consequently, the level of killing varies from one experiment to another, as does the ability of IL2 to enhance the killing in both normal and IL2-depleted cell cultures.

An example of a killing assay performed with 660 TIL shortly after antigen stimulation is shown in Fig. 4A. The tumor target cells were first coated with the fusion protein or with ch14.18 antibody and then used as targets in a 7-hr ⁵¹Cr-release assay. At the higher effector/target ratio (50:1), the antibody alone stimulated killing, but to a much lesser extent than CH3–IL2. The effect of CH3–IL2 was more pronounced with TIL that had been deprived of IL2 for 4 days.
A similar experiment comparing the fusion protein and exogenously added IL2 was performed 1 week later, when the autologous killing activity had declined. For this reason the duration of the cytotoxicity assay was extended to 16 hr. As seen in Fig. 4B, the addition of IL2 (100 units/ml) to the assay mixture had little effect. The stimulatory effect of CH3-IL2 in these experiments was quite striking, especially at the lower effector/target ratios and when IL2-depleted effector cells were used. The results were less pronounced once the TIL had been cultured without antigen stimulation with 660 mel tumor cells (data not shown). In all cases, the amount of stimulation obtained by coating the tumor cells exceeded that obtained by adding equivalent levels of IL2.

**DISCUSSION**

A fusion protein consisting of an intact tumor-specific chimeric antibody and human IL2 (CH3-IL2) has been shown to retain both antibody and IL2 functions. IL2 activity was measured by the ability of the fusion protein to stimulate the proliferation of resting human and mouse T cells. Constructs containing smaller portions of the antibody molecule were also found to retain full IL2 activity. These results contrast with an earlier report (20) in which a purified Fab-IL2 fusion protein was 200-fold less active than recombinant IL2 in a proliferation assay. Our constructs also differ from those reported by Fell et al. (20) in that we have directly fused antibody and IL2 sequences, without the introduction of artificial linker residues. In the case of our 14.18 Fab-IL2, we could not demonstrate antigen binding, but this was not likely due to the fusion of IL2, since a similar construct made with the V regions of B72.3 maintained both IL2 and antigen binding activities. Genetically engineered 14.18 Fab was also found to have greatly reduced antigen-binding activity.

Melanoma cells expressing GD2 that have bound the CH3-IL2 fusion protein serve as much better targets for cytotoxic T cells. This was demonstrated by using a TIL line (660 TIL) that had been maintained in culture in the presence of high concentrations of IL2 with periodic antigen stimulation by autologous melanoma cells (660 mel). The stimulatory effect was most pronounced when the TIL had been maintained without IL2 for several days. The amount of IL2 that would be bound to the 660 mel cells under saturating conditions would be equivalent to 50 units/ml in the assay, if these cells express >10^7 GD2 sites as was reported for M21 melanoma cells (8). These levels of IL2 added to the assay mixture were less effective than the fusion protein in stimulating cytolytic activity.

In the experiment depicted in Fig. 4A, there was some enhancement of killing of cells coated with chimeric antibody alone at the 50:1 effector/target ratio, suggesting some ADCC activity. However, the 660 TIL line does not contain detectable numbers of cells expressing Fc receptors as shown by immunofluorescence analyses (unpublished data).

The ability to combine the targeting of a tumor-specific antibody together with a potent cytokine such as IL2 should prove useful in directing and localizing its effect at tumor sites. In this regard, tumors secreting either IL2, tumor necrosis factor, or granulocyte colony-stimulating factor after transfection with genes encoding these molecules are rejected upon transplantation into syngeneic animals due to the establishment of cellular immunity (21-23). Antibody targeting of cytokines may achieve this same end by stimulating a cytotoxic T-cell response and in this way augment the helper T-cell function that may be lacking in cancer patients.
The use of a whole antibody–IL2 fusion protein may have advantages over antibody fragment–IL2 fusion proteins, since additional effector functions (ADCC and CDC) will be targeted to the same site. As discussed earlier, experiments using peripheral blood cells from patients treated with IL2 have already shown that the ch14.18 antibody can mediate ADCC against tumor cells (12). In this case the primary effector cells are Fc receptor-positive (CD16’) natural killer cells. The work presented here suggests that cytotoxic T cells can also be stimulated to kill autologous antigen-positive cells that have been coated with the antibody–IL2 fusion protein.

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