Antitumor Effects of a Bispecific Antibody Targeting CA19–9 Antigen and CD16

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ABSTRACT

Bispecific murine monoclonal antibodies that target tumor and FcγRIII (CD16) can promote relevant tumor lysis by large granular lymphocytes. For these antibodies to be clinically useful, their properties should be maintained in vivo, where competing human immunoglobulin, shed target antigen, and shed CD16 may be encountered. At a minimum, bispecific antibody antitumor effects should be preserved in whole blood. Furthermore, potentiation of tumor lysis should be reflected by demonstrating the ability of bispecific antibody-retargeted effector cells to infiltrate and mediate lysis of organized tumor. If these characteristics are demonstrated, and there is evidence of in vitro efficacy of bispecific antibody-based therapy in a relevant animal model, further clinical development of such antibodies would be warranted. In this report the ability of CL158 bispecific antibody supernatants to mediate lysis of SW948 tumor growing in monolayer is shown to be preserved in the presence of interleukin 2-activated whole blood. When SW948 cells were grown in vitro as multicellular human tumor spheroids, incubation with interleukin 2-activated lymphocytes (LAK cells) and CL158 led to structural and widespread necrosis. This was dependent on CL158 and resistant to competition by pooled human immunoglobulin or interleukin 2-exposed whole blood. These effects were not promoted by the monospecific antibodies produced by the parent clones of CL158 and were not observed when the IgG2a variant of CA19–9 antibody, which mediates conventional antibody-dependent cellular cytotoxicity, was used instead of its bispecific derivative. To examine the efficacy of bispecific antibody-based treatments on in vitro tumor, scid mice bearing early s.c. SW948 xenografts were treated with interleukin 2 for 5 consecutive days, supplemented by three i.v. injections of 10^7 human LAK cells and various antibodies. Treatment of mice bearing SW948 tumors with LAK cells did not retard tumor growth, but when CL158 was added, significant delays in tumor growth were observed. Tumor growth delay required treatment with both LAK cells and the bispecific antibody. Treatment with the IgG2a variant of CA19–9 antibody, alone or with LAK cells, had no effects on tumor growth. Although the mechanisms of these antitumor effects require further study, it is clear that human LAK cell treatment of animals bearing early, established s.c. tumors is enhanced by the addition of bispecific antibodies with relevant binding characteristics. When compared with the IgG2a isotype variant of CA19–9 monoclonal antibody, this bispecific antibody offers the advantages of preservation of activity in physiological conditions, infiltration and disruption of organized tumor in vitro, and antitumor effects in a relevant xenograft model. These characteristics support the continued clinical development of bispecific antibodies with specificity for tumor and FcγRIII.

INTRODUCTION

Monoclonal antibody therapy of human malignancies has led to infrequent clinical responses (1, 2). The mechanisms underlying these responses remain speculative and include direct growth inhibition by signal transduction effects (3, 4), complement-mediated cytotoxicity (5), induction of antidiotopic cascades (6), and ADCC mediated by polymorphonuclear leukocytes (7), mononuclear phagocytes (8), or LGLs (9–11). Although ADCC is a potent mechanism of tumor lysis, this in vitro property has not been observed either in animal models of successful antibody therapy or in clinically responding patients.

To be successful, ADCC requires that antibodies bind to tumor antigens via Fab domains and to cellular Fcγ receptors via their Fc domains. Thus, this mechanism is inhibited if effector cell Fcγ receptors are engaged by other antibodies. Since human serum contains high concentrations of IgG antibody, expression of clinically relevant ADCC may be inhibited. To circumvent this obstacle, bispecific antibodies have been constructed to simultaneously target tumor antigens and various effector cell trigger molecules other than the immunoglobulin-binding domain of Fcγ receptors (12). Such molecules have been prepared as chemically linked antibody heteroconjugates (13–15) or as bispecific monoclonal antibodies derived by somatic cell hybridization of two existing hybridomas (16–20). Diverse tumor antigens have been targeted, while the effector cell targets usually have been elements of the T-cell receptor/CD3 complex. Fcγ receptor epitopes outside the immunoglobulin Fcγ-binding domain also are attractive effector cell targets, since cytotoxicity may be triggered by binding to these epitopes (21). Bispecific antibodies targeting tumor and the high-affinity Fcγ receptor expressed by polymorphonuclear leukocytes and mononuclear phagocytes (FcγRI) have been prepared and shown to be potent inducers of relevant tumor lysis (14).

To redirect the potent cytotoxicity mediated by LGLs, we have prepared a bispecific monoclonal antibody which binds to the tumor antigen CA19–9 and FcγRIII (CD16), the Fcγ receptor for aggregated immunoglobulin that is expressed by LGLs, differentiated macrophages, and some T-cells (22). This antibody potentiates lysis of relevant tumor by LAK cells, enriched for LGLs, in the presence of competing human immunoglobulin. If this type of antibody is to be useful in the therapy of neoplastic diseases, its properties must be preserved in whole blood, in the presence of competing immunoglobulin and shed FcγRIII. Furthermore, the ability of bispecific antibody redirected relevant effector cells to infiltrate organized tumor must be demonstrated. Finally, the efficacy of this therapeutic approach should be demonstrated in an appropriate animal model.

MATERIALS AND METHODS

Antibodies. CL158 bispecific antibody was produced by fusion of the CA19–9 and 3G8 hybridomas, as described (22). CA19–9 antigen is expressed by a variety of adenocarcinomas (23), and FcγRIII is expressed by large granular lymphocytes, macrophages, and polymorphonuclear leukocytes (24). The supernatants of CL158 used in these experiments have properties similar to those of bispecific antibody partially purified by ion exchange chromatography of concentrated CL158 supernatants. Quantitation of murine immunoglobulin in the supernatants was performed by enzyme-linked immunosorbassay, as described (22). Chromatographic analysis of the CL158 supernatants

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3 The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; LGL, large granular lymphocyte; IL-2, interleukin 2; LAK cells, IL-2-activated lymphocytes; MHTS, multicellular human tumor spheroid.
demonstrated that the supernatants are typically composed of immunoglobulin containing 30–35% bispecific, 45–50% CA19–9, and 15–25% 3G8 antibodies (22). The concentrations of CL158 reported in the text refer to total murine IgG and not the estimated fraction that is bispecific. CA19–9 IgG1 isotype hybridoma was obtained from the American Type Culture Collection (Rockville, MD). CA19–9 IgG2a antibody was a kind gift of Dr. Zenon Steplewski, Wistar Institute (Philadelphia, PA). 3G8 hybridoma was the kind gift of Dr. Jay Unkelless, Mt. Sinai School of Medicine (New York, NY).

Cells. Cells of the SW948 line were obtained from the American Type Culture Collection. These cells express CA19–9 antigen and are known to be targets for CL158-promoted lysis by IL-2-activated human lymphocytes. These cells were maintained in L-15 medium (Gibco, Grand Island, NY) supplemented with penicillin/streptomycin (JRH Bioscience, Lenexa, KS) and 10% fetal bovine serum (Bioproducts for Science, Indianapolis, IN). Cells were grown in T-175 culture flasks (Nunc, Naperville, IL) and used for cytotoxicity assays and inoculation into mice when in log growth phase.

**Generation of Human LAK Cells.** Effector cells were obtained from normal donors. Lymphocytes were purified from whole blood by Ficoll-Isoaque (Ficoll-Paque, Pharmacia, Uppsala, Sweden) density centrifugation, and monocytes were then removed by adherence to plastic. The nonadherent cell suspension was incubated in RPMI 1640 containing supplemental glutamine (Gibco) and 10% fetal bovine serum containing 1000 units/ml recombinant IL-2. Recombinant IL-2 was generously provided by Cetus, Inc. (Emeryville, CA) (10). We have shown that these cells (LAK cells) contain 10–20% large granular lymphocytes and can promote lysis of allogenic tumor that is augmented by relevant bispecific monoclonal antibodies (22). In some experiments, whole blood was anticoagulated with 10 units/ml heparin (Heparin Sodium, Hynomomed, Rosemont, IL) and incubated in flat-bottomed 96-well plates (Costar, Cambridge, MA) in 1000 units/ml recombinant IL-2 prior to use in cytotoxicity assays.

**Cytotoxicity Assays.** Monolayer cultures of SW948 cells were labeled with 51Cr as described (10). Fifty µl (106) of labeled cells were aliquoted into wells containing 100 µl whole blood and various antibodies and incubated in a humidified 37°C incubator. Supernatants were sampled for radioactivity content at 4 h. Spontaneous release of label was less than 30% in all assays. Cytotoxicity was calculated by the formula:

\[
\% \text{ Lysis} = \frac{\text{ER} - \text{SR}}{\text{TC} - \text{SR}} \times 100
\]

where ER is experimental release, SR is spontaneous release, and TC is total counts. A minimum of two assays contributed to each depicted experimental value.

To determine cytotoxicity in MHTS, 200–400 µµl spheroids were incubated overnight with 111In-oxine and then added to 17 × 100 mm polystyrene tubes containing 2 ml of IL-2-activated effector cells or heparinized whole blood activated in IL-2 for 36 h. After 24 h, the specific lysis of cells in the MHTS was calculated using the formulas described above. Equal volumes (0.2 ml) of MHTS were added to each sample of whole blood.

**MHTS Cultures.** The SW948 (colon adenocarcinoma) cell line was obtained from the American Type Culture Collection (Rockville, MD). These cells were trypsinized from monolayer cultures and resuspended as single cells in Leibovitz (L-15) medium containing 10% heat-inactivated fetal bovine serum. The MHTS were cultured in 75-cm² flasks coated with 1% agarose. Flasks were incubated at 37°C in a humidified atmosphere for 10 days or until spheroids reached an appropriate size (200–400 µµm). Spheroid cultures were supplemented with fresh media weekly.

**Coculture of MHTS with LAK Cells and Antibodies.** MHTS (200–400 µµm) were cultured with LAK cells alone or with LAK cells in the presence of various antibodies. LAK cells were resuspended to 2 × 10⁶ cells/ml in L-15 medium containing 1000 units/ml of recombinant IL-2. Two hundred fifty µl (5 × 10⁵) LAK cells were added to each 17 × 100 mm polystyrene tube containing a packed MHTS volume of 100 µ (approximately 10⁶ tumor cells) in media containing 1000 units/ml recombinant IL-2. When CL158 was used, 100 µl of a 1:100 dilution of culture supernatant containing the bispecific antibody was included in SW948 MHTS-containing tubes, yielding a final bispecific antibody concentration of 7 ng/ml. In experiments using IL-2-activated whole blood, the same volume of SW948 MHTS was added directly to tubes containing the blood. Tubes were placed on a rocker platform for 24 h at 37°C. Samples were then harvested and analyzed by morphology and immunohistochemistry as described below.

**Histology and Immunohistochemistry of MHTS.** Spheroids were pelleted, washed in phosphate-buffered saline, fixed in 2.5% glutaraldehyde for 30 min, and embedded in 3% agar. Some pellets were dehydrated in graded alcohols and embedded in paraffin. Sections (5 µµm) were cut and stained with hematoxylin and eosin for histological examination. Antigen expression in the spheroids was studied by immunoperoxidase staining using the Vectastain ABC kit as described (25).

Lymphocyte infiltration into multicellular spheroids was studied by the immunoperoxidase technique. Sections (5 µµm) were stained with mouse antibodies PD7/26/16 and 2B11, directed against human leukocyte common antigen (DAKO Corporation, Carpenteria, CA). The slides were developed with diaminobenzidine and counterstained with hematoxylin before examination. Immunofluorescence was used to evaluate lymphocyte infiltration into SW948 MHTS by labeling LAK cells with Leu 19-PE (anti-CD56 phycoerythrin conjugate; Becton-Dickenson, Mountain View, CA) for 30 min, removing unconjugated antibody by washing, and then coincubating the labeled LAK cells with SW948 MHTS for 4 h. Samples were analyzed by fluorescent microscopy using a Nikon UFX-II instrument (26).

Histological evaluation was performed by studying a minimum of 12 spheroids/experimental group. Grading of histological damage was evaluated by determining the percentage of each examined MHTS demonstrating necrosis. The average percentage of necrosis in the examined spheroids in a given experimental group was determined and reported as follows: 76–100% as Grade 4; 51–75% as Grade 3; 26–50% as Grade 2; 1–25% as Grade 1; and no damage as Grade 0. All samples were read by at least two independent observers in blinded fashion.

**Animal Studies.** CB17/ICR acid mice were used in these studies. These immunodeficient mice do not have functional T- and B-cell lymphocytes due to defective VDJ recombinase but possess intact natural killer activity. Mice were maintained in microisolator cages in the Fox Chase Cancer Center Laboratory Animal Facility. On day 1, mice received s.c. inoculations of 1.0 × 10⁷ SW948 tumor cells in the flanks. Tumor growth was spherical and was measured with Vernier calipers. Mice were followed at least twice weekly and sacrificed when tumor diameters reached 2.0 cm.

Treatment commenced on day 4. When used, LAK cells, antibodies, and IL-2 were administered via tail vein injections on days 4, 6, and 8, while IL-2 was also given i.p. on days 5 and 7. In these studies, each agent was used at the following doses per treatment: 1 × 10⁷ LAK cells; 5 × 10⁶ units IL-2; and 10 ng CL158 in a total volume of 100 µµl media. Each treatment group contained at least four mice. In each experiment a single batch of LAK cells from a single donor was used to treat all animals in all treatment groups. Effector cells were verified to possess killing activity. Mice were maintained in microisolator cages in the Fox Chase Cancer Center Laboratory Animal Facility. On day 1, mice received s.c. inoculations of 1.0 × 10⁷ SW948 tumor cells in the flanks. Tumor growth was spherical and was measured with Vernier calipers. Mice were followed at least twice weekly and sacrificed when tumor diameters reached 2.0 cm.

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**RESULTS**

**Preservation of Bispecific Antibody Effects in Whole Blood.** Fig. 1 demonstrates the cytotoxic lysis of SW948 cells growing in monolayer by heparinized human whole blood activated in IL-2. Cytotoxicity rose to nearly 40% after blood had been
BISPECIFIC ANTIBODY ANTITUMOR EFFECTS

Fig. 1. Promotion of 31Cr labeled SW948 cell lysis by CL158 bispecific monoclonal antibody. Whole blood, treated with heparin, was incubated in 1000 units/ml recombinant IL-2 for indicated periods as described in text in microtiter wells. Supernatants were harvested 4 h after the addition of 31Cr-labeled SW948 cells. D, no added antibody; E, 10 ng/ml CL158 added prior to addition of targets; H, 1 µg/ml CA19-9 IgG2a isotype monoclonal antibody.

Table 1 Cytotoxicity of SW948 cells and MHTS by LAK cells and bispecific antibody CL158

<table>
<thead>
<tr>
<th>Target</th>
<th>Percentage cytotoxicity</th>
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<tbody>
<tr>
<td>SW948 cells</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>34.1 ± 3.0*</td>
</tr>
<tr>
<td>SW948 spheroids</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>24 h</td>
<td>18.0 ± 1.1</td>
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* Effectortarget ratio = 25:1.

Table 1 shows that tumor lysis by LAK cells, measured by 111In release, was specifically enhanced by CL158. Tumor in monolayers was lysed efficiently after 4 h of exposure to effector cells, whereas lysis of tumor in spheroids required a 24-h incubation. In all experiments using 24-h incubations, tumor lysis from SW948 spheroids was at least doubled by the addition of CL158 to LAK cells.

Fig. 2. Effects of LAK cells and bispecific antibody CL158 on SW948 MHTS. MHTS were grown to 200–400-µm size and incubated with LAK cells and CL158 as described in the text. A, MHTS incubated with human LAK cells (1:5:1 effectortarget ratio) for 24 h. × 100. B, MHTS incubated with human LAK cells plus 7 ng/ml CL158 supernatant for 24 h. H & E, × 100. Inset, B field at × 40.

Histological and Immunohistochemical Characterization of LAK Cell Infiltration of MHTS. Histological examination of SW948 spheroids incubated with LAK cells alone showed minimal necrosis and destruction of spheroid integrity (Fig. 2A). In contrast, addition of bispecific antibody to LAK cells resulted in cell damage in the MHTS at 4 h. After 24 h of incubation, the spheroids were 75–85% necrotic, with necrosis distributed throughout the spheroids (Fig. 2B). In some experiments, lymphocyte infiltration initially was distributed throughout the periphery of the MHTS, while in others, the periphery was focally breached, with central necrosis accompanied by a rim of viable-appearing tumor cells.

Coculture of LAK cells with SW948 MHTS resulted in the minimal infiltration of lymphocytes, assessed by immunohistochemistry, after 24 h. When SW948 MHTS were incubated with LAK cells and 7 ng/ml CL158, superficial cellular infiltrates were demonstrated within 4 h of incubation, while more extensive lymphocyte infiltration was evident after 24 h. Immunofluorescent staining of LAKs infiltrating SW948 MHTS.
in the presence of CL158 demonstrated that CD56-expressing cells were components of the infiltrating lymphocyte population (data not shown).

To study the specificity of the enhancement effect of CL158 bispecific antibody when LAK cells were cocultured with SW948 spheroids, control antibodies were tested under identical culture conditions. These included the parent antibodies of the CL158 hybrid hybridoma, CA19–9 and 3G8, IgG1 isotype antibodies which do not promote ADCC. The IgG2a class switch mutant of CA19–9 also was used, since it has identical tumor specificity to CL158 and can promote ADCC. As shown in Table 2, when SW948 MHTS were incubated with LAK cells and CA19–9 or 3G8, no histological damage was observed. Incubation of SW948 spheroids with LAK cells and the IgG2a variant of monoclonal antibody CA19–9 resulted in some necrotic cells randomly distributed within the MHTS, with preservation of the general morphological appearance of the spheroids. When SW948 MHTS were incubated with bispecific antibody and LAK cells, a variety of histological changes were observed, ranging from total MHTS disintegration to more intermediate degrees of damage. These changes were not observed when SW948 MHTS were incubated with CL158 in the absence of LAK cells. Extensive damage to SW948 MHTS was also observed when they were cocultured with LAK cells and CL158 in the presence of human serum (15% v/v). When the SW948 MHTS were incubated with heparinized, IL-2-activated whole blood, lysis augmentation was also preserved.

Fig. 3. Effects of human LAK cells and CL158 bispecific monoclonal antibody in ascites mice bearing SW948 human tumor xenografts. Mice inoculated with 1×10^7 SW948 cells treated with human LAK cells and CL158 as described in text. ■, CL158 therapy alone; ○, LAK cell therapy alone; ●, LAK plus CL158 therapy.

**DISCUSSION**

Bispecific antibody CL158 previously has been shown to promote lysis of CA19–9-containing tumor by effector cells expressing FcγRIII. Its major advantages over a conventional monospecific antibody with identical tumor specificity have been its efficiency in promoting tumor lysis at low antibody concentrations or low effector:target ratios, together with its supernatants to human LAK cells significantly inhibited tumor growth in animals bearing early, established SW948 s.c. tumor. Both LAK cells and CL158 were required to see this effect; neither treatment alone inhibited tumor growth.

A variety of treatment permutations were examined to demonstrate the specificity of the CL158 effect (Table 3). Only the combination of LAK cells plus CL158 caused significant reductions in SW948 tumor growth. The IgG2a isotype variant of CA19–9 is known to promote ADCC (22). Treatment with the IgG2a isotype CA19–9 antibody alone, or with human LAK cells, failed to retard tumor growth, even though this antibody was used at relatively high doses of 1 μg/treatment/animal. In contrast, nanogram doses of CL158 had no effect on SW948 tumor growth, while these same doses were effective in combination with human LAK cells. Since these data were obtained using CL158 clonal supernatants, it is possible that an unexpected component of the supernatant was responsible for these observations, even though non-antibody-secreting CL158 variant supernatants in combination with human LAK cells had no effect on tumor growth.

**Table 2: Histological damage to tumor spheroids by LAK cells and antibodies**

| Antibodies | No LAK cells | LAK cells | LAK cells + 15% (v/v) human serum | Whole blood
|------------|--------------|-----------|---------------------------------|--------------
| SW948 spheroids | 0 | 1 | 1 | 0
| None | 0 | 1 | 1 | 0
| 3G8 (IgG1) | 0 | 1 | 1 | 0
| CA19–9 (IgG1) | 0 | 1 | 1 | 0
| CA19–9 (IgG2a) | 0 | 1 | 1 | 0
| CL158 (IgG1) | 0 | 1 | 1 | 0

* Based on visual estimates of percentage of necrosis of MHTS compared to untreated controls, as follows: Grade 0, none; 1, <25%; 2, 26–50%; 3, 51–75%; 4, 76–100%.

Human peripheral blood lymphocytes activated in vitro for 72–96 h in 1000 units/ml recombinant IL-2 were used at effector:target ratios of approximately 0.5:1.

Whole blood from normal donors was heparinized and incubated for 36 h in 1000 units/ml IL-2. MHTS were then aliquoted into suspension and incubated for 18 h.

Final antibody concentrations: 3G8 and CA19–9 (IgG1), 1 μg/ml; CA 19–9 (IgG2a), 3 μg/ml; CL158, 7 ng/ml.

* Not tested.
The whole-blood studies were undertaken to investigate the influence of possible inhibitory factors interfering with lysis such as circulating FcyRIII (5 nm) or CA19–9 antigen (≥70 units/ml when positive), or enzymatic degradation of the bispecific antibody. Although the levels of these antigens or activity were not directly measured, there was no evidence of tumor lysis inhibition. It is possible that mechanisms other than conjugation of LAK cells to tumor contributed to tumor lysis. For example, differentiated human macrophages express FcγRIII and mediate relevant tumor lysis promoted by CL158. However, the incubation conditions used here did not lead to induction of FcγRIII by these cells (data not shown). Also, it has been shown that activated large granular lymphocytes secrete granulocyte-macrophage colony-stimulating factor when activated, leading to the induction of cytotoxicity by polymorphonuclear leukocytes (29). While such a scenario is possible, the morphological appearance of the cells infiltrating MHTS exposed to IL-2-activated whole blood was that of lymphocytes, consistent with previously documented mechanisms of tumor lysis promotion by CL158.

It cannot be concluded that preservation of CL158 effects in whole blood demonstrates the clinical relevance of this class of antibody. Many other factors, such as antibody biodistribution, relevant effector cell trafficking to tumor, and the activation state of migratory effectors, must be considered in the development of bispecific antibodies. However, these data show that CL158 effects are preserved in a physiological milieu which inhibits cytotoxicity promotion by most conventionally structured monospecific antitumor monoclonal antibodies. Potent antitumor effects may be anticipated if the above-noted obstacles can be overcome.

Cytotoxicity assays that measure radioactivity release assess tumor lysis capability but do not address the ability of cytotoxic effector cells to infiltrate and mediate destruction of solid tumors. MHTS have been used extensively as in vitro models to characterize the effects of radiation on organized tumor possessing poorly oxygenated, necrotic cores (30). More recently, spheroids have been used to characterize radiolabeled antibody penetration and binding (31). The use of MHTS targets has provided valuable insight into the cytotoxic potential and mechanisms by which cells activated in IL-2 (LAK cells) bind to and induce the necrosis of cells growing in organized tumor (32–34). We have evaluated the contribution of CL158 to LAK cell-mediated destruction of tumor cells growing in multicellular spheroids. CL158 has been shown to significantly augment tumor infiltration by LAK cells, inducing substantially more leukocyte infiltration and necrosis than do LAK cells alone. Jääskeläinen et al. (33) have shown that LAK cells infiltrate into spheroids derived from human glioma cell lines as a frontier. However, infiltration has varied according to the glioma cell line used, with evidence of tumor cell damage at sites that were distant from the infiltrating front of lymphocytes. Iwasaki et al. (32) have performed similar studies using a different human glioma spheroid model. They too demonstrated substantial cytotoxicity after 24 h. In contrast, peripheral blood lymphocytes did not affect spheroid growth. The infiltrative leukocytes appeared to attach directly to tumor cells, with formation of intracytoplasmic granules in the effectors.

In the present study, the effects of LAK cells on tumor spheroids were less striking in that there was minimal leukocyte infiltration or tumor cell necrosis after 24 h of incubation. Since SW948 spheroids possess typical adenocarcinoma morphology and contain numerous desmosomes and other junctions, it is possible that the infiltration of leukocytes is more restricted than in the glioma spheroids. Experiments to further correlate leukocyte infiltration with expression of junctional complexes by the spheroids are required to address this possibility. To achieve the results seen with the addition of CL158, longer periods of incubation may be required to see equivalent degrees of leukocyte infiltration, tumor cell lysis, and destruction of spheroid morphology when LAK cells are used alone. The promotion of MHTS infiltration and tumor destruction by the addition of CL158 to human LAK cells demonstrates a potentially clinically useful characteristic of this type of bispecific antibody. Since the effects were not as striking when the IgG2a isotype variant of CA19–9 was substituted for CL158, the MHTS system provides further demonstration of the superior ability of the bispecific construct to promote relevant cell conjugation and lysis. The maintenance of this superiority in whole blood is similarly encouraging evidence of the potential clinical applicability of bispecific antibodies targeting tumor and FcγRIII.

Treatment of scid mice bearing early established tumor xenografts with human IL-2-activated lymphocytes and IL-2, using the doses and schedules described in this report, did not delay the growth of these tumors. However, the addition of relevant bispecific monoclonal antibodies to this treatment regimen led to significant growth delays and, in some instances, cures. These observations are striking in that the substitution of significantly higher doses of the IgG2a isotype variant of CA19–9 for CL158 was not effective, even though this variant mediates in vitro ADCC. In other experiments, the parent antibodies of CL158, CA19–9 IgG1 variant and 3G8, have had no effects on xenograft growth (data not shown).

The murine model used in these studies differs from those used in conventional investigations of antibody treatment effects on the growth of human xenografts because the bispecific antibodies used here interact with human CD16 and do not bind to murine splenocytes or peritoneal macrophages. Accordingly, it was necessary to treat the tumor-bearing mice with human lymphocytes. The particular treatment permutations, doses, and schedules were chosen to permit the testing of conditions identified as being optimal in the in vitro studies. Thus, IL-2-activated LAK cells were used, as was IL-2 therapy of the

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Median days to 2.0-cm-diameter tumor</th>
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<tbody>
<tr>
<td>No human LAK cells</td>
<td>40 (5)*</td>
</tr>
<tr>
<td>No antibody</td>
<td>32 (5)</td>
</tr>
<tr>
<td>CA19–9 (IgG2a)</td>
<td>28 (5)</td>
</tr>
<tr>
<td>10⁷ human LAK cells</td>
<td></td>
</tr>
<tr>
<td>No antibody</td>
<td>27 (9)</td>
</tr>
<tr>
<td>CL158</td>
<td>62 (9)</td>
</tr>
<tr>
<td>CA19–9 (IgG2a)</td>
<td>38 (5)</td>
</tr>
<tr>
<td>Control supernatant</td>
<td>35 (5)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, no. of treated animals.

** Longer survival than other treatments (P < 0.05 by log-rank and Wilcoxon).
mice to maintain the proliferative and cytotoxic effects of IL-2 on the administered human cells. In these studies, the hypothesis was tested that franked human effector cells would be endowed with tumor specificity, which would provide them with superior in vivo antitumor effects (12). Other strategies, such as therapy with peripheral blood LGL or tumor-binding doses of antibody, will be of interest in determining the optimal therapeutic routes of bispecific monoclonal antibody administration. The therapeutic extrapolation of the strategy utilized in this report would involve mixing the bispecific antibody with autologous, leukopheresed mononuclear cells and readministering the antibody-franked cells. While the quantitative trafficking of such cells to tumor may be limited, the data presented here suggest that therapeutically significant outcomes are seen when the tumor volume is small. In contrast, a substantial excess of the IgG2a isotype variant does not have the in vivo effects seen with CL158. While the use of higher CA19–9 doses may have produced therapeutic effects, the dose was chosen to provide a control for treatment with very low doses of CL158. Since there is no circulating murine immunoglobulin in scid mice, other manipulations will be required to confirm the relevance of in vitro data, demonstrating that preservation of effector:target interactions in the presence of competing immunoglobulin is a major advantage of bispecific antibodies over their monospecific antitumor counterparts.

The efficacy of CL158 in treating early tumor is perhaps best explained by its promotion of effector:target cell interactions in vivo, leading to tumor destruction and growth inhibition. Very low concentrations of CL158, added to LAK cells prior to injection into tumor-bearing mice, were sufficient to inhibit tumor growth, while antibody supernatants alone were not sufficient to see this effect. These data suggest that the CL158-franked effectors were better able to bind to in vivo tumor. Since scid mouse splenocytes and macrophages do not interact with CL158, it is unlikely that the observed effects can be attributed to CL158-mediated interactions between these cells and the human tumor. Also, these effects cannot be attributed to rejection of tumor by human lymphocytes that have engrafted in scid mice. Fewer than 5% of scid mouse splenocytes are human CD3-positive 4 weeks after i.p. injection of 1 × 10^7 human lymphocytes (35). In these studies, within 4 weeks, most animals inoculated with SW948 tumor had 2-cm-diameter tumors. Furthermore, i.v. injection of human lymphocytes does not promote their engraftment in scid mice (35). Finally, no antitumor effects were observed in mice treated with human LAK cells alone in the present study.

Using Winn assays and intraperitoneal models, antitumor effects have been observed following treatment with bispecific antibodies which bind tumor and CD3 (36–38). These intraperitoneal models offer advantages over the subcutaneous model used in this study in that they permit easier study of effector:target interactions and do not require effector cell migration from the vasculature. Treatment-induced infiltration of established tumor was not described in these studies or observed in this report. This raises the possibility that effective treatment requires small tumor volumes, where it will be technically difficult to demonstrate relevant effector cell infiltration of tumor.

It may prove difficult to demonstrate that the ability of bispecific antibodies to promote the infiltration and destruction of MHTS can be recapitulated in vivo using xenograft models requiring therapy with human effectors. Since a restricted number of human cells can be injected, the treatment of mice bearing macroscopic tumors will always use a suboptimal number of effectors. Thus, the MHTS data are best interpreted as demonstrating a biological attribute of bispecific antibody-directed cells. Studies with syngeneic models of bispecific antibody therapy will be required to address the hypothesis that such antibodies can promote tumor infiltration by cytotoxic effectors and to critically examine the influence of factors such as vascular supply, hydrostatic pressure, constraints on transendothelial migration of effectors, and cytokine-regulated trafficking of these cells.

Irrespective of the mechanisms of effect, it is clear that a brief course of bispecific antibody-based therapy had significant effects on the growth of early, established, subcutaneously implanted tumor. These findings provide impetus for further investigation of this class of antibodies. Many questions regarding optimization of doses, schedule, and the limits imposed by tumor burden and target antigen expression must be considered, along with the exploration of treatment effects and tumor infiltration properties of human LAK cell subpopulations, so that the critical components of this treatment model can be understood. These investigations will supplement further studies that address the mechanisms of action of therapy and describe how these effects compare to the well-described in vitro cytotoxicity properties of bispecific antibodies.

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