Characterization of a Novel Bispecific Antibody That Mediates Fcγ Receptor Type I-dependent Killing of Tumor-associated Glycoprotein-72-expressing Tumor Cells

Christina Russoniello, Chezian Somasundaram, Jeffrey Schlom, Yashwant M. Deo, and Tibor Keler

Medarex, Inc., Annandale, New Jersey 08801 [C. R., C. S., Y. M. D., T. K.]; and Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland 20892 [J. S.]

ABSTRACT

A bispecific antibody was made by chemical conjugation of Fab' fragments from humanized antibodies specific for tumor-associated glycoprotein-72 (TAG-72) and high-affinity immunoglobulin receptor, Fcγ receptor type I (FcγRI). The purified anti-TAG-72 × anti-FcγRI (HCC49×H22) bispecific antibody had an approximate Mₐ of 111,000, consistent with a F(ab')₂, and bound specifically to KLEB and LS174T tumor cell lines, which express the TAG-72 tumor antigen. Furthermore, HCC49×H22 was shown to simultaneously bind to KLEB cells and a soluble FcγRI fusion protein, demonstrating the bifunctional nature of the molecule. Using IFN-γ-treated monocytes as effector cells, concentrations of the bispecific antibody in the range of 1–10,000 ng/ml mediated specific lysis of TAG-72-positive tumor cells. In contrast, the bispecific antibody did not promote antibody-dependent cellular cytotoxicity of a cell line that was negative for TAG-72 antigen. Importantly, the antibody-dependent cellular cytotoxicity activity of the bispecific antibody was significantly greater than that of the monoclonal antibody HCC49. These in vitro data indicate that the humanized bispecific antibody HCC49×H22 has the appropriate specificity and functional activity for further evaluation as potential immunotherapy for TAG-72-positive malignancies.

INTRODUCTION

TAG-72 is a high molecular weight mucin that is expressed in a variety of malignancies, including gastrointestinal carcinoma, non-small cell lung carcinoma, gastric pancreatic carcinomas, and breast, ovarian endometrial, and prostatic carcinomas (1–5). In normal adult tissues, TAG-72 is detected in greatly reduced amounts or not at all, with the exception of secretory endometrium (5). TAG-72 has been identified on 85% of colorectal adenocarcinoma, 70% of breast tumors, and 95% of ovarian cancers (1). Several mAbs specific for TAG-72 have been well characterized in vitro (6, 7), in animal models (8, 9), and in clinical trials for diagnosis and therapy of a variety of carcinomas. In particular, the mAb CC49 has demonstrated excellent imaging of human tumors, and the preliminary results from Phase III clinical trials of radioimaging-guided surgery of colorectal cancer are very encouraging (10). In contrast to the diagnostic success, the therapeutic regimes investigated with radiolabeled murine CC49 have not demonstrated any significant efficacy thus far (11–13). However, recent results on the use of murine mAb CC49 in combination with IFN-γ and other potential therapeutics have shown encouraging results. These studies were conducted with the murine form of the CC49 mAb and necessitated the use of only one or two unique injections of antibody due to anti-immunoglobulin responses. To reduce the potential human antimusone antibody response, mAb CC49 was humanized by grafting of the mouse hypervariable regions to a human IgG1 backbone (9). The humanized CC49 (HCC49) retained the efficient and selective tumor reactivity of the parent mAb. The potent imaging characteristics and expected low immunogenicity of HCC49 provide the rationale for developing this mAb for immunotherapy as a bispecific antibody.

Bispecific antibodies with specificity for a tumor antigen and a cytotoxic trigger molecule on immune cells provide an effective means to recruit the antitumor activity of effector cells (14). The human FcγRs act as cytotoxic trigger molecules when expressed on the appropriate effector cells (15). The high-affinity receptor for IgG, FcγRI, unlike the lower-affinity FcγRII and FcγRIII, is expressed primarily on monocytes and macrophages, and its expression can be induced on granulocytes with granulocyte colony stimulating factor or IFN-γ (16, 17). Thus, the cellular distribution of FcγRI is restricted primarily to immune cells that can mediate ADCC and phagocytosis. The anti-FcγRI mAb 22 binds to a site distinct from the Fc ligand-binding region and can trigger FcγRI effector functions in the presence of saturating IgG (15, 16, 18). The murine mAb 22 was recently successfully humanized for clinical applications (19). Directing tumor cells to FcγRI with bispecific molecules targeted to various tumor antigens has resulted in efficient ADCC and phagocytosis of relevant tumor cell lines in vitro (20–23). Two such bispecific antibodies, MDX-H210 (anti-HER2/neu × anti-FcγRI) and MDX-447 (anti-EGFR × FcγRI) are currently being evaluated in Phase I/II clinical trials.

Here, we describe a new bispecific antibody made from the humanized anti-TAG-72 mAb HCC49 and the humanized anti-FcγRI mAb H22. This chemically linked bispecific antibody,
HCC49×H22, was evaluated for specificity of binding to TAG-72- and FcγRI-bearing cells. Furthermore, we have demonstrated monocytes effectively mediated ADCC of TAG-72-expressing tumor cells in the presence of low concentrations of bispecific antibody. These preclinical results support further development of HCC49×H22 bispecific antibody for the treatment of TAG-72-expressing cancers.

MATERIALS AND METHODS

Antibodies and Cell Lines. The U-937 histiocytic lymphoma, CEM and Jurkat T lymphoblastoid, A431 epidermoid carcinoma, and LS174T colon adenocarcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). The endometrial carcinoma cell line KLEB (13) was provided by J. Schlam (National Cancer Institute, Bethesda, MD). Cell lines were grown in RPMI supplemented with 10% FCS at 37°C in a humidified incubator with 5% CO2. The HCC49, H22, and H425 mAbs were purified from hybridoma culture supernatants using protein A affinity chromatography (Pharmacia, Piscataway, NJ).

Preparation of HCC49×H22. Bispecific antibodies were prepared according to modified techniques of Brennen (24). In brief, F(ab')2 fragments of mAbs HCC49 and H22 were made by pepsin digestion. The F(ab')2 fragments were purified by consecutive protein A affinity and size exclusion chromatographic techniques. The H22 F(ab')2 fragments were then reduced with 0.5 mM DTT at 30°C. The reduced fragments were then treated with 5,5'-dithiobisnitrobenzoic acid at room temperature. The H22 Fab'-TNB derivative was purified by G25 gel filtration. The HCC49 F(ab')2 fragments were reduced with 15 mM mercaptoethanolamine at 30°C and purified by G25 gel filtration. Equimolar amounts of H22 Fab'-TNB and HCC49 Fab'-SH were mixed and incubated overnight at 4°C. The bispecific conjugate was purified from the uncoupled Fab' fragments by gel filtration chromatography. The anti-EGFR bispecific antibody, MDX-447 (H425×H22), was prepared in the same manner as was HCC49×H22. The preparation of the anti-Her2/neu antibody, MDX-H210 (52OC9×H22), was described previously (23). Routine endotoxin testing on the bispecific antibodies showed endotoxin levels to be <0.1 endotoxin unit/ml.

Gel Electrophoresis. Samples of HCC49 F(ab')2, H22 F(ab')2, and HCC49×H22 were evaluated by gel electrophoresis on a 4–15% Tris-glycine gel (Bio-Rad, Hercules, CA) at 5 μg protein per lane. Gels were stained with Coomassie Blue destained and analyzed on a Sun Sparcstation IPC densitometer (Millipore, Marlborough, MA).

Flow Cytometry. Cell lines were washed with PBS containing 0.1% BSA and added to 96-well plates at 3–105 cells/well. The bispecific antibody HCC49×H22 and controls were diluted in PBS containing 0.1% BSA and 0.05% sodium Azide and added to wells in an equal volume. After 90 min at 4°C, the cells were washed, and PE-conjugated antihuman-IgG was added (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA). Following incubation at 4°C for 60 min, the cells were washed and suspended in 1% paraformaldehyde in PBS. For the bifunctional flow cytometry assay, KLEB cells were incubated with HCC49×H22, HCC49 F(ab')2, or soluble FcγRI at 4°C for 60 min. Cells were washed and then incubated with soluble FcγRI (4°C for 90 min) in the form of supernatant from transfected COS cells expressing a soluble fusion protein con-
**Fig. 3** Dose curve of cell surface binding of HCC49×H22 and HCC49 mAb to KLEB cells. KLEB cells were incubated with HCC49×H22 (●) or HCC49 mAb (○) at 4°C for 90 min. Bound HCC49×H22 or HCC49 mAb was detected with antihuman IgG-PE. Cells were fixed and analyzed on FACScan. Data points, mean fluorescence of duplicate determinations from a representative experiment.

**Fig. 4** Simultaneous binding of HCC49×H22 to TAG-72 on KLEB cells and FcγRI by bifunctional flow cytometry assay. HCC49×H22 (●), HCC49 F(ab')2 (○), or FcγRI μ fusion protein (▼) were incubated with KLEB cells at 4°C for 90 min. Cells were washed and incubated with supernatant containing soluble FcγRI μ fusion protein at 4°C for 90 min. Bound FcγRI was detected with antihuman IgM PE (1:50), and cells were fixed and analyzed on FACScan. Data points, mean fluorescence of duplicate determinations from a representative experiment.

consisting of the extracellular domain of FcγRI and human IgM heavy chain (a gift from Dr. Brian Seed, Massachusetts General Hospital, Boston, MA). The cells were washed and resuspended in 1% paraformaldehyde in PBS. Samples were analyzed on Becton Dickinson FACScan.

**Experiments to Evaluate Up-Regulation of TAG-72 on LS174T Cells Grown in Mice.** LS174T cells were grown in immunosuppressed mice (severe combined immune deficient mice or FVB/N mice irradiated with 650 rad) by s.c. inoculation of 7.5 × 10⁶ cells. LS174T cells were allowed to develop into tumors for 9 days. Tumors were dissected out and dissociated by passing through a wire mesh. Adherent cells were cultured for 1 week, and then both the LS174T cells derived from the tumor and the original LS174T cell line maintained in culture were stained with HCC49×H22 or 520C9×H22. Staining was performed as described for previous flow cytometry experiments.

**ADCC.** Monocytes were obtained from different donors as normal adult source leukocytes (Advanced Biotechnologies Inc., Columbia, MD). Monocytes were isolated by Ficoll Hypaque (Pharmacia) density gradient, followed by cold aggregation. Monocytes were cultured for 24–48 h in Teflon jars with macrophage serum-free medium (Life Technologies, Inc., Grand Island, NY). IFN-γ (1000 units/ml; Genzyme, Cambridge, MA) was added to up-regulate FcγRI expression. Just prior to the assay, monocytes were washed and adjusted to 10⁷/ml. Targets were labeled with 100 mCi of ⁵¹Cr for 1 h, washed well to remove excess ⁵¹Cr, and adjusted to a concentration of 10⁷/ml. Dilutions of antibodies were prepared in medium and added together with effector and targets cells to microtiter plates in a final volume of 150 μl/well. Incubation times for ADCC ranged from 14 to 18 h, and then 50-ml aliquots of supernatant were analyzed for radioactivity in a gamma counter. Cytotoxicity was calculated by the formula: % lysis = [(experimental cpm – target leak cpm)/(detergent lysis cpm – target leak cpm)] × 100%. Specific lysis is calculated by subtracting the percentage lysis without antibody from the percentage lysis with antibody. All samples were tested in triplicate. Statistical analysis was performed by Student’s t test and was calculated by SIGMAPLOT software (Jandel Corporation, San Francisco, CA). P < 0.05 was considered significant. All experiments were performed a minimum of two times.

**RESULTS**

**Biochemical Characterization of HCC49×H22.** The electrophoretic patterns of HCC49×H22 and the F(ab')₂ fragments of HCC49 and H22 were evaluated under nonreducing and reducing conditions. Under nonreducing conditions (Fig. 1A), the major band (~70%) of the bispecific antibody HCC49×H22 had an apparent Mr of 111,000, consistent with the molecular weights of the HCC49 F(ab')₂ (Mr 112,000) and H22 F(ab')₂ (Mr 113,000). Approximately 20% of bispecific preparation migrated as Mr 85,000 and Mr 22,000 species, which most likely represent dissociation of one light chain from the bispecific antibody. This dissociation appears to be induced by the SDS-PAGE because high-performance liquid chromatography analysis with a TSK3000 column demonstrated that >99% of the bispecific antibody eluted as a single peak consistent with the size of a F(ab')₂ (data not shown). Furthermore, because the chemical linkage used to make HCC49×H22 resulted in a disulfide linkage [presumably at the hinge region, as in F(ab')₂ fragments], under reducing conditions, HCC49×H22 was completely dissociated to fragments corresponding to Fab' heavy and light chains (Fig. 1B). These fragments coincided with those generated from the HCC49 F(ab')₂ and H22 F(ab')₂.

**Binding to Cell Lines Expressing TAG-72.** HCC49×H22 demonstrated specific binding to TAG-72-bearing cell lines in flow cytometry assays. HCC49×H22 at concentrations of 0.1 μg/ml and above bound to cell lines known to express...
TAG-72 (KLEB, >80% positive; and LS174T, >75% positive) but did not show any significant binding to TAG-72-negative A431 cells (Fig. 2). The observed binding was mediated exclusively by the HCC49 arm of the bispecific molecule because H22 F(ab')2 was negative for binding on the TAG-72-expressing cell lines. The relatively broad distribution of cell-associated fluorescence after staining with HCC49×H22 (Fig. 2) indicates heterogeneous binding to individual cells. The bispecific and mAb demonstrated similar binding kinetics to KLEB cells (Fig. 3), although the absolute fluorescence with HCC49 was greater than that with HCC49×H22. This apparent difference in binding may be accounted for by the expected lower avidity of the monovalent bispecific antibody compared to the bivalent mAb. Alternatively, the antihuman IgG probe used for detection may have reacted better with the mAb than with the bispecific antibody.

The bispecific nature of HCC49×H22 was demonstrated by simultaneous binding to TAG-72 on KLEB cells and soluble FcyRI-IgM chimeric molecule. In the bispecific flow cytometry assay, TAG-72-bearing cells bound with HCC49×H22 were incubated with the soluble FcyRI-IgM protein and detected with antihuman IgM labeled with PE. The results presented in Fig. 4 demonstrate a dose-dependent binding of antihuman IgM-PE to KLEB cells, indicating that the bispecific antibody bound to TAG-72 could engage FcyRI. The fusion protein alone with KLEB cells and the HCC49 F(ab')2 with KLEB cells did not generate a mean fluorescence intensity greater than background. In addition, a control cell line that does not express TAG-72 (CEM cells) did not show a mean fluorescence intensity greater than background in these experiments (data not shown).

**Binding to FcyRI-positive U-937 Cells.** The binding of HCC49×H22 to cellular FcyRI was tested using the monococyte-like cell line U-937, which expresses high levels of this receptor when it is cultured with IFN-γ. This cell line was used because primary monocyte cultures maintain high levels of human IgG, which would interfere with the detection of this humanized bispecific antibody. HCC49×H22 demonstrated dose-dependent binding to U-937 cells that saturated at ~1 μg/ml (Fig. 5). The F(ab')2 fragment of HCC49 was unable to bind to U-937 cells, and similarly, the HCC49×H22 binding was abrogated in the presence of excess murine mAb 22. These results demonstrated that the bispecific antibody bound specifically to FcyRI.

**ADCC of TAG-72-expressing and Nonexpressing Cell Lines.** Having demonstrated the appropriate biochemical and binding characteristics for HCC49×H22, ADCC assays were performed to evaluate the functional activity of the bispecific antibody to mediate killing of TAG-72-expressing tumor cell lines by monocytes. Cultured monocytes have been reported to down-regulate FcyRI expression and function (25); therefore, monocytes were cultured with IFN-γ prior to ADCC assay. Monocytes were washed to remove IFN-γ, and dilutions of HCC49×H22 (●) or HCC49 (□) mAb were prepared at various concentrations in media. KLEB cells were labeled with 51Cr and added with antibodies to the monocytes. The final E:T was 100:1, and cultures were incubated for 18 h. Supernatants were collected by apheresis, enriched by cold aggregation, and incubated for 24 h with IFN-γ prior to ADCC assay. Supernatants were then harvested, and percentage specific lysis was determined as described in "Materials and Methods." Non-antibody-dependent lysis in these assays was 36 ± 5.6%. Columns, means of five experiments; bars, SE. Statistical significance was determined using paired Student’s t test; Ps indicated above each concentration represent analysis of HCC49×H22 versus HCC49 mAb.
individual cell lines. Importantly, A431 cells that overexpressed EGFR but were negative for TAG-72 were not lysed in the presence of HCC49×H22. However, in the presence of an anti-EGFR bispecific antibody (H425×H22), the A431 cells were efficiently lysed, indicating that the effector cells used could kill A431 cells through FcγRI (Fig. 7). Because KLEB cells express both EGFR and TAG-72, both bispecific antibodies were able to mediate equivalent lysis of these cells. Furthermore, simply engaging the trigger molecule FcγRI with H22 F(ab')2 did not induce any significant lysis of either A431 cells or KLEB cells. These data suggest that HCC49×H22 effectively mediates specific killing of TAG-72-bearing cells.

**DISCUSSION**

We have constructed a bispecific antibody, HCC49×H22, that specifically binds to and mediates the killing of TAG-72-positive tumor cells via the myeloid trigger molecule FcγRI. Both the HCC49 (9) and H22 (19) antibodies have been extensively characterized in vivo and in vitro regarding their specificity for TAG-72 and FcγRI, respectively. HCC49 is currently being evaluated in Phase III clinical trials for imaging TAG-72-negative target cells in an 18-h assay. Supernatants were then harvested, and percentage specific lysis was determined as described in “Materials and Methods.” Columns, means of triplicate determinations from a representative experiment; bars, SD.

**Table 1** ADCC activity of HCC49×H22 correlates with binding to TAG-72

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>% ADCC</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLEB</td>
<td>30 ± 7 (4)</td>
<td>1625 ± 743 (4)</td>
</tr>
<tr>
<td>Jurkat</td>
<td>17 ± 5 (4)</td>
<td>704 ± 183 (4)</td>
</tr>
<tr>
<td>LS174T</td>
<td>9 ± 2 (3)</td>
<td>325 ± 87 (3)</td>
</tr>
<tr>
<td>A431</td>
<td>2 ± 2 (3)</td>
<td>12 (2)</td>
</tr>
</tbody>
</table>

ADCC activity was measured by 51Cr-release assay with IFN-γ-stimulated monocytes at an E:T of 100:1. The values represent the mean of the specific (antibody-dependent) lysis ± SE at 1.0 μg/ml HCC49×H22. The number of experiments is indicated in parentheses.

MFI, mean fluorescence intensity; measured by flow cytometry after staining at 4°C with 1 μg/ml HCC49×H22 and goat antihuman IgG-PE probe. The values represent the mean ± SE. The number of experiments is indicated in parentheses.

from Phase I/II clinical trials with a structurally comparable bispecific antibody, H425×H22 (humanized Fab' × humanized Fab', without cross-linker), has shown very low human anti-bispecific antibody responses. On the other hand, the murine Fab' × humanized Fab' bispecific antibody S209×H22 has generated significant anti-bispecific titers in some patients in a comparable clinical setting after multiple administrations (27). The method of coupling used to generate HCC49×H22 may be susceptible to reduction in serum, however, our experience with H425×H22 has shown that bispecific antibodies constructed with a reducible cross-linker have serum stability and pharmacokinetic profiles that are similar to those of bispecific antibodies made with a thioether linkage.

The in vitro characterization of HCC49×H22 was partially impeded by the fact that few cultured cell lines retain stable high expression of TAG-72 (29). Furthermore, relevant cell lines that express TAG-72, such as KLEB and LS174T, appear to have an unequal distribution of the antigen on individual cells, as represented by the broad histogram patterns in the flow cytometry analysis. Previous reports have demonstrated that CC49 (Fab')2 mediates excellent tumor imaging in animal models, supporting the observation that TAG-72 is expressed to a higher level and more uniformly in vivo than in vitro (30). Using HCC49×H22, we have found an ~30% greater binding to LS174T cells passaged in immuno-suppressed mice compared to the same cells cultured in vitro. The increased antigen expression was specific for TAG-72, because in these same cells Her2/neu expression was not affected by passage through the mouse (Fig. 8). Despite the lack of ideal target cells, this bispecific antibody mediated significant killing of three TAG-72-positive tumor cell lines. The results obtained with KLEB cells (which express the highest level of TAG-72) are comparable to those previously reported with other bispecific antibodies that are currently being evaluated in clinical trials (23). Importantly, the percentage of ADCC mediated by HCC49×H22 correlated with...
2242 Bispecific Antibody Lysis of TAG-72-expressing Cells

Fig. 8 Enhanced expression of TAG-72 on LS174T cells grown in a mouse. Expression of TAG-72 was analyzed by flow cytometry. The LS174T cells that grew as tumors in immunosuppressed mice (□) were compared to LS174T cells maintained in culture (∆). A, TAG-72 expression on cells stained with HCC49×H22 (1 μg/ml). B, Her2/neu expression on cells stained with 520C9×H22 (1 μg/ml). Incubations were done for 90 min at 4°C. Bound HCC49×H22 or 520C9×H22 was detected with antihuman IgG-PE. Cells were fixed and analyzed on FACScan. Columns, mean fluorescence of duplicate determinations from a representative experiment.

the level of target cell binding by the bispecific antibody, and TAG-72 negative A431 cells were not lysed in an antibody-dependent fashion.

Comparison of HCC49×H22 with HCC49 demonstrated that the bispecific antibodies had greater ADCC activity than the mAb, particularly at very low concentrations. This may be explained by the fact that HCC49×H22 binds the FcγRII outside of the IgG binding site, whereas HCC49 mAb has to compete with serum IgG already present on the receptors. A similar study showed that bispecific MDX-H210 mediated better ADCC than did the corresponding mAb (520C9-human IgG1 chimera) in the presence of human IgG (28, 31). These data demonstrate that, at low concentrations, H22-targeted bispecific antibodies can circumvent the antibody blockade to recruit monocytes for ADCC more efficiently than a corresponding mAb.

These preclinical data support the further development of this bispecific antibody for therapy of TAG-72-positive cancers. It may be of particular value to investigate the activity of this bispecific antibody in combination with specific cytokines. IFN-γ may be of particular interest because this cytokine has demonstrated the ability to up-regulate TAG-72 expression on tumor cells and FcγRI-mediated monocyte activities. TAG-72 expression can be increased by IFN-γ on selected cultured tumor cell lines, and more importantly, enhanced expression on human adenocarcinomas has been shown in clinical trials (32–34). Furthermore, HCC49×H22-mediated ADCC was significantly greater when monocytes were cultured in the presence of IFN-γ (6.0 ± 3% without IFN-γ; 27.5 ± 1.5% with IFN-γ; n = 3; P < 0.05). Currently, a combination therapy of 520C9×H22 (anti-Her2/neu bispecific antibody) with IFN-γ is being investigated in a Phase I clinical trial. We are currently investigating the effect of selected cytokines on the antitumor activity of HCC49×H22.

REFERENCES
18. Anderson, C. L., Guyre, P. M., Whitten, J. C., Ryan, D. H., Looney, R. J., and Fanger, M. W. Monoclonal antibodies to Fe receptors for IgG on human mononuclear phagocytes: antibody characterization and in-

Downloaded from clincancerres.aacrjournals.org on July 12, 2017. © 1998 American Association for Cancer Research.


Characterization of a novel bispecific antibody that mediates Fcγ receptor type I-dependent killing of tumor-associated glycoprotein-72-expressing tumor cells.

C Russoniello, C Somasundaram, J Schlom, et al.