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Antibody Targeting to a Class I MHC-Peptide Epitope Promotes Tumor Cell Death

Vaughan P. Wittman,*† David Woodburn,† Tiffany Nguyen,‡ Francisca A. Neethling,† Stephen Wright,‡ and Jon A. Weidanz1*†

Therapeutic mAbs that target tumor-associated Ags on the surface of malignant cells have proven to be an effective and specific option for the treatment of certain cancers. However, many of these protein markers of carcinogenesis are not expressed on the cells’ surface. Instead these tumor-associated Ags are processed into peptides that are presented at the cell surface, in the context of MHC class I molecules, where they become targets for T cells. To tap this vast source of tumor Ags, we generated a murine IgG2a mAb, 3.2G1, endowed with TCR-like binding specificity for peptide-HLA-A*0201 (HLA-A2) complex and designated this class of Ab as TCR mimics (TCRm). The 3.2G1 TCRm recognizes the GVL peptide (GVLPALPQV) from human chorionic gonadotropin β presented by the peptide-HLA-A*0201 complex. When used in immunofluorescent staining reactions using GVL peptide-loaded T2 cells, the 3.2G1 TCRm specifically stained the cells in a peptide and Ab concentration-dependent manner. Staining intensity correlated with the extent of cell lysis by complement-dependent cytotoxicity (CDC), and a peptide concentration-dependent threshold level existed for the CDC reaction. Staining of human tumor lines demonstrated that 3.2G1 TCRm was able to recognize endogenously processed peptide and that the breast cancer cell line MDA-MB-231 highly expressed the target epitope. The 3.2G1 TCRm-mediated CDC and Ab-dependent cellular cytotoxicity of a human breast carcinoma line in vitro and inhibited in vivo tumor implantation and growth in nude mice. These results provide validation for the development of novel TCRm therapeutic reagents that specifically target and kill tumors via recognition and binding to MHC-peptide epitopes. The Journal of Immunology, 2006, 177: 4187–4195.

The MHC class I (MHC-I)² system, known as the HLA class I system in humans, can be thought of as nature’s proteomic scanning chip because it continuously processes intracellular protein, generating peptides for presentation on the surface of cells. Many cancer cells display tumor-specific peptide-HLA complexes derived from processing of inappropriately expressed or overexpressed proteins, called tumor-associated Ags (1–3). These Ags are the focus of a number of approaches to cancer therapy including various vaccine technologies and adoptive T cell transfer regimens (4–6). Recent success using Abs for the treatment of breast cancer (trastuzumab) and non-Hodgkin lymphoma (rituximab) has provided new impetus for identifying the next generation of tumor targets for Ab therapy. The majority of proteins produced by a cell reside within intracellular compartments, thus preventing their direct recognition by Ab molecules. The abundance of intracellular proteins that is available for degradation by proteasome-dependent and independent mechanisms yields an enormous source of peptides for surface presentation in the context of the MHC-I system (7). A new class of Abs that specifically recognizes HLA-restricted peptide targets (epitopes) on the surface of cancer cells would significantly expand the therapeutic repertoire if it could be shown that they have anti-tumor properties which could lead to tumor cell death.

Abs have recently been identified that recognize peptides in the context of MHC in a way that mimics the specificity of a TCR (8–10). These Abs recognize tumor-associated peptides derived from the Ags expressed within the cell and on its surface (e.g., gp100, telomerase (hTERT), MUC1, MAGE, and NY-ESO-1) in the context of HLA-A1 or A2 (8–11). The majority of these Abs were isolated from bacteriophage libraries as Fab (8, 9, 11) and have not been examined for anti-tumor activity because they do not activate innate immune mechanisms (e.g., complement-dependent cytotoxicity (CDC)) or activate Ab-dependent cellular cytotoxicity (ADCC). Demonstration of anti-tumor activity is critical as therapeutic mAbs are thought to act through several mechanisms which engage the innate response, including Ab or complement-mediated phagocytosis by macrophage, CDC and ADCC (12–16). For the current study we generated an HLA-peptide specific mAb, which we called a TCR mimic (TCRm), specific for a peptide from the tumor-associated Ag human chorionic gonadotropin β-chain (hCGβ) in the context of HLA-A2.

hCG is a member of the glycoprotein hormone family that shares homology with luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone. Each of these is a heterodimer with a variable β-chain and a common α-chain. hCG is most commonly associated with pregnancy assessment but is also a marker for tumors resulting from tissues associated with placenta or germ cells. In a comprehensive review of hCG in cancer, Stenman et al. (17) reported that hCGβ is found in the serum of 45–60% of patients with biliary and pancreatic cancers, and 10–30% of other cancers. Immunohistochemical analysis and urinalysis
have been used to demonstrate the presence of hCGβ in lung, gynecological, and head and neck cancers. The aggressiveness and resistance to therapy of bladder cell carcinoma expressing hCGβ has been associated with an autocrine anti-apoptotic effect elicited by the free β-chain (18). A series of Abs that bind hCG were developed for use as diagnostic reagents and hCGβ-specific Abs, which have application in pregnancy testing as well as monitoring for hCG positive tumors continue to be developed (19). An anti-hCGβ vaccine (for use in treatment of human cancer) that targets hCGβ to dendritic cells has been shown to elicit both cytotoxic and helper T cell responses to peptide pulsed target cells and tumor cell lines (20). Recently, several MHC-I epitopes from hCGβ have been identified which bind with high affinity to HLA-A*0201 molecules (21).

A first step in evaluating the efficacy of therapeutic Abs is in vitro assessment of their specificity and ability to induce tumor cell lysis via the activation of complement and ADCC. The therapeutic successes of the mAbs trastuzumab and rituximab are thought to be due, at least in part, to their ability to promote ADCC and CDC (15, 22–24). In this report, we investigate the Ag binding specificity, in vitro lytic capabilities and in vivo tumor growth inhibition of a TCRm mAb, 3.2G1, which is specific for the GVL peptide (residues 47–55 from hCGβ) presented in the context of HLA-A2.

Materials and Methods

Cell lines and Abs

The human tumor cell lines T2 (lymphoblast), SW620 (colon), MDA-MB-231 (breast) and BT20 (breast) were obtained from the American Type Culture Collection. The SKOV3.A2 (ovarian) transfectant was a gift from Dr. V. Ramakrishna (CellDiv Therapeutics). The hybridoma cell line BB7.2 that produces the BB7.2 mAb (specific for the HLA-A2) was a gift from Dr. R. Buchli (Pure Protein). The murine IgG2a and IgG1 isotype control Abs UPC-10 and MOPC 21, respectively, were purchased from Sigma-Aldrich. Other anti-murine Abs that were used for analysis or ELISA were purchased as part of a mouse Ig panel or the SBA Clonotyping System/HIRP from Southern Biotechnology Associates. Goat anti-mouse secondary Abs used for cell staining included the Fcγ-specific FITC-labeled F(ab')2 and the Fcγ (1 + 2a + 2b + 3)-specific PE-labeled Ig from Jackson ImmunoResearch Laboratories. The murine IgG2a and IgG1 isotype control Abs UPC-10 and MOPC 21, respectively, were purchased from Sigma-Aldrich. Other anti-murine Abs that were used for analysis or ELISA were purchased as part of a mouse Ig panel or the SBA Clonotyping System/HIRP from Southern Biotechnology Associates. goat anti-mouse secondary Abs used for cell staining included the Fcγ-specific FITC-labeled F(ab’)2, and the Fcγ (1 + 2a + 2b + 3)-specific PE-labeled Ig from Jackson ImmunoResearch Laboratories. The murine IgG2a and IgG1 isotype control Abs UPC-10 and MOPC 21, respectively, were purchased from Sigma-Aldrich. Other anti-murine Abs that were used for analysis or ELISA were purchased as part of a mouse Ig panel or the SBA Clonotyping System/HIRP from Southern Biotechnology Associates.

Cell culture

Cell culture medium included IMDM and RPMI 1640 from Cambrex, L-15 from Mediatech, and FlexiBME from Invitrogen Life Technologies. Medium supplements included heat-inactivated FBS and penicillin/streptomycin from Sigma-Aldrich and L-glutamine from HyClone. Recombinant human IL-2 was obtained from PeproTech. All tumor lines were maintained in culture medium containing glutamine, penicillin/streptomycin, and 10% FBS. Cell cultures were maintained at 37°C in 5% CO2 atmosphere, with the exception of MDA and SW620, which were cultured without CO2. MDA and SW620 cells were cultured in l-15, SKOV3.A2, and T2 in IMDM, and BT20 in RPMI 1640 medium. When necessary, attached cells were released from flasks using TrypLE Express (Invitrogen Life Technologies). Human PBMC from anonymous donors were obtained from separation cones of discarded apheresis units from the Coffee Memorial Blood Bank (Amarillo, TX) after platelet harvest. Cells were separated on a Ficoll gradient, then washed, counted, and resuspended in AIM-V medium containing 200 U/ml IL-2 per a concentration of 2-2.5 × 105 cells/ml. PBMC were maintained at this concentration with medium changes and addition of IL-2 every 2–3 days for a maximum of 7 days. These conditions have been shown to maintain and activate resident NK cells within the PBMC population (25). Murine hybridoma cells were initially grown in RPMI 1640 medium supplemented with 10% FBS, glutamine, and penicillin/streptavidin (RPMI/10) as described below. After selection for binding specificity, clones were grown in RPMI/10 to provide supernatant containing the Abs of interest or in serum-free medium to provide supernatant for isolation of purified Abs from protein G columns (GE Healthcare BioSciences).

Pepitides and HLA-A2 complexes

The following peptides were synthesized at the Molecular Biology Resource Facility, University of Oklahoma (Oklahoma City, OK.): KIFGSLAFL (residues 369–377, designated Her-2), eukaryotic initiation translation factor 4 γ VLMTEDIKL (residues 720–728, designated elf4G), human choriionic gonadotropin-β TMTVRQLGVQ (residues 40–48, designated TMT), VLGVLPAL (residues 44–53, designated VLG), and GVLFALPQV (residues 47–55, designated GVL). HLA-A2 extracellular domain and β2m were produced as inclusion bodies in Escherichia coli and refolded essentially as described previously (26). After refolding, the peptide-HLA-A2 mixture was concentrated and properly folded complex was isolated from contaminants on a Superdex 75 sizing column (GE Healthcare BioSciences AB). This complex, designated the monomer, was biotinylated using the BirA biotin ligase enzyme (Avidity) and purified on the P3X63.Ag8.653 myeloma cell line using a Clonacell-HY Kit (StemCell Technologies). After 2 wk in semisolid medium, single clones were picked, transferred to 96-well tissue culture plates, and grown for 3–4 days in Clonacell Medium E. Supernatants were tested for the appropriate mAb production by ELISA as described below.

ELISA

ELISA was performed using Maxisorb 96-well plates (Nunc). Assays to evaluate binding specificity of the TCRm Abs were done on plates coated with either HLA-A monomer (50 ng per well) or HLA tetramer (100 ng/well). Bound Abs were detected with peroxidase-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) followed by ABTS (Pierce). Reactions were quenched with 1% SDS. Absorbance was measured at 405 nm on a Victor II plate reader (PerkinElmer). The SBA clonotyping system/HRP and mouse Ig panel from Southern Biotechnology Associates was used to estimate the concentration of 3.2G1 (isotype IgG2a) in the supernatant of FBS-containing medium. The assay was run according to manufacturer’s directions, and 3.2G1 signal was compared with that of an IgG2a standard supplied by the manufacturer. Development, quenching, and analysis of the plate were performed as described above for the TCRms.

Cell staining

T2 cells were pulsed with the peptides at 20 μg/ml for 4 h in growth medium, with the exception of the peptide-titration experiments in which the peptide concentration was varied as indicated. Cells were washed and resuspended in staining buffer (SB) (PBS plus 0.5% BSA plus 2 mM EDTA) and then stained with 1 μg of 3.2G1, BB7.2, or isotype control Ab for 15–30 min in 100 μl of SB. Cells were then washed with 3 ml SB and the pellet was resuspended in 100 μl of SB containing 2 μl of either of two goat anti-mouse secondary Abs (FITC or PE labeled). After incubating for 15–30 min at room temperature, the wash was repeated, and cells were resuspended in 0.5 ml SB, analyzed on a FACScan instrument (BD Biosciences) and evaluated using Cell Quest software (BD Biosciences). Tumor cell lines were stained and evaluated in the same manner as the T2 cells after being released from plates and washed in SB. Tetraper comepetition stains were conducted in the same order described above, except that tetramer at the appropriate concentration was mixed with the Ab and allowed to stand for 40 min before the mix was added to the cells.

Cytotoxicity analysis

Specific cell lysis in the CDC, NK cell, and ADCC assays was evaluated using the Cytotox 96 nonradioactive cytotoxicity lactate dehydrogenase (LDH) assay (Promega) following the instructions provided by the manufacturer. This assay measures the release of cellular LDH into the culture supernatant after cell lysis. All cells were grown or pulsed with peptide in
their appropriate growth medium, but final incubations of cells in the presence of complement (CDC) or human PBMCs (NK and ADCC) was conducted in AIM-V medium for 4 h at 37°C. CDC analysis of T2 cells took place under three different conditions: the Ab concentration was varied and competing or noncompeting tetramer added (1), peptide mixes were used to pulse cells (2), or GVL peptide was titrated for use in cell pulsing (3). CDC analysis of MDA-MB-231 cells using Ab dilutions and tetramer competition was conducted on adherent cells. Exact conditions are described in the figure legends and/or Results. Standard complement or LoTox complement was obtained from Cedarlane Laboratories. All cells used as targets for cytotoxicity assays were pulsed with 4 h with peptide. Specific lysis in the CDC assays was calculated as follows: \((E - T + \text{Ab release})/(E - T + \text{Maximum release - spontaneous release})\) × 100 = specific release. ADCC reactions using human PBMC effector cells were conducted on MDA-MB-231 target cells using 3.2G1 or W6/32 Abs at a final concentration of 10 μg/ml. E/T ratios were varied as indicated in the figures. NK analysis was performed by mixing human E with K562 cells and incubating as above. Specific lysis in ADCC analysis was calculated as follows: \((E + T + \text{Ab release} - E + T + \text{Ab release})/(E - T + \text{Maximum release - spontaneous release})\) × 100 = specific release. Specific lysis in NK analysis was calculated: \((E + T + \text{Ab release} - E + T + \text{Maximum release - spontaneous release})\) × 100 = specific release. Spontaneous and maximum release were measured before and after, respectively, lysis of target cells with 0.9% Triton X-100.

**In vivo studies**

Athymic nude mice (CBByLC-Foxn1–/–) were obtained from The Jackson Laboratory and housed under sterile conditions in barrier cages. Each of 19 mice was implanted with 5 × 10^6 freshly harvested (97% viable) MDA-MB-231 cells in Matrigel (Sigma-Aldrich). Mice received an i.p. injection of 250 μl of either 3.2G1 or isotype control Ab weekly for the first 6 weeks after the appearance of the last tumor in the isotype control group (a total of 4189 μg of either 3.2G1 or isotype IgG 2a control Ab). There is a clear relationship between Ab concentration and staining intensity of the pulsed cells.

**Generation of TCRm Abs specific for the HLA-A2-GVL peptide complex**

BALB/c mice were immunized and boosted with HLA-A2 complexes prepared by refolding E. coli-expressed HLA-A2 and β2m in the presence of the hCGβ peptide located at 47–55 aa (designated GVL). Following the second boost, mice were sacrificed and splenocytes were isolated and used in a standard hybridoma fusion protocol. After growth in semisolid medium, clones were transferred to individual wells and cultured, and supernatant was collected. GVL or not pulsed with peptide. Staining was conducted, and the MFI at 0.01 μg/ml VLQ peptide and nonpulsed cells were used as negative controls. After pulsing and the addition of Ab, cells were stained and analyzed. The MFI of cells stained with the 3.2G1 Ab titrated over a range from 10 to 150 MFI, whereas there was much less variation with BB7.2 staining, which ranged from 250 to 350 MFI (Fig. 1D). We conclude from these findings that 3.2G1 staining intensity is dependent on the density of the specific epitope on the surface of cells.

**Results**

**Characterization of the TCRm Ab 3.2G1**

To establish that the 3.2G1 TCRm mAb isolated in the initial screening was HLA-A2 restricted and peptide-specific, a series of assays to characterize its binding specificity were performed. The first assessment used refolded peptide/HLA-A2 molecules as targets for testing the 3.2G1 TCRm in an ELISA. Fig. 1A shows the results of ELISA analysis of supernatant from hybridoma 3.2G1 vs HLA-A2/β2m complex refolded with its cognate peptide GVL or with one of three other irrelevant peptides. Significant reactivity was seen only in wells containing the GVL tetramer, indicating the TCR-like specificity of the Ab. Coating of each well was confirmed by ELISA using the HLA-A2 conformation-specific Ab BB7.2 (data not shown).

To confirm the specificity of 3.2G1 TCRm for the GVL/A2 complex on the surface of T2 cells, the cells were pulsed with the specific peptide GVL, with irrelevant peptides VLQ or TMT, or with no peptide, and then stained with 3.2G1 (Fig. 1B). The concentration of 3.2G1 in supernatant was determined by isotype-specific ELISA, and the Ab was used at 1 μg per stain. Binding to the surface of the cells was detected with goat anti-mouse FITC labeled secondary Ab, and the cells were analyzed by flow cytometry. The GVL-pulsed cells shifted significantly (mean fluorescence intensity (MFI) of 141), compared with cells pulsed with the irrelevant peptides containing closely related sequences VLQ and TMT or no peptide (MFI of 7.3, 7.5, and 9.0, respectively).

A correlation between Ab concentration and level of staining of peptide-pulsed cells was established by titration of the Ab (Fig. 1C). 3.2G1 Ab was diluted over a range of 0.01 to 3 μg and then used to stain T2 cells that had been either pulsed with 20 μg/ml GVL or not pulsed with peptide. Staining was conducted, and the MFI was determined by subtracting the no-peptide MFI value from the MFI of GVL-pulsed cells. The staining reactions appeared to saturate with 3.2G1 at ~1 μg/100 μl and retained the ability to differentiate GVL-pulsed cells from those that were not pulsed down to 0.01 μg. The MFI at 0.01 μg of Ab was 14.3, compared with 388 for 1 μg of Ab. There is a clear relationship between Ab concentration and staining intensity of the pulsed cells.

To assess the effect of peptide-HLA density on the cell surface on 3.2G1 TCRm staining, T2 cells were next pulsed with varying levels of GVL peptide. The peptide was serially diluted and added to cells at concentrations ranging from 50 to 0.1 μg/ml VLQ peptide and nonpulsed cells were used as negative controls. After pulsing and the addition of Ab, cells were stained and analyzed. The MFI of cells stained with the 3.2G1 Ab titrated over a range from 10 to 150 MFI, whereas there was much less variation with BB7.2 staining, which ranged from 250 to 350 MFI (Fig. 1D). We conclude from these findings that 3.2G1 staining intensity is dependent on the density of the specific epitope on the surface of cells.

**Competition studies using tetramer constructs containing either the GVL or VLQ peptide were conducted to evaluate the fine specificity of binding of Ab 3.2G1 (Fig. 1E). Preincubation of 3.2G1 with the GVL tetramer inhibited the final staining of GVL pulsed T2 cells in a concentration-dependent manner with 50% inhibition occurring at roughly 0.07 μg of tetramer/μg of Ab. There was essentially no inhibition of staining by the VLQ tetramer at any of the concentrations tested which were up to 40-fold higher than the concentration of GVL tetramer required for 50% inhibition. These findings strongly support the conclusion that the 3.2G1 TCRm mAb specifically binds to its cognate epitope GVL/A2 on the surface of T2 cells.

**CDC using 3.2G1 Ab**

Murine IgG2a Abs have been found to efficiently direct CDC, whereas the IgG1 isotype does not (27). This fact and the corresponding ability of this isotype to bind human FcR (see below) led to our selection of the 3.2G1 TCRm mAb. T2 cells pulsed with various peptides were used as targets for the initial 3.2G1-directed CDC analysis, because they could easily be loaded to a high density with any of a number of peptides. The effect of the relative
density of the appropriate peptide-A2 complex on the surface of T2 cells was probed by pulsing with GVL, TMT, a mixture of the two, or no peptide while holding the Ab concentration constant at 2.5 μg/ml. Fig. 2A illustrates the CDC results of cells pulsed with various ratios of peptide (GVL to TMT) for both the HLA-A2-specific BB7.2 Ab and 3.2G1. BB7.2 is a murine IgG2b Ab and this isotype also efficiently fixes complement (27). BB7.2-driven lysis demonstrates that there is little difference between cells pulsed with peptides at the various concentrations. The lysis of nonpulsed cells is lower, as might be expected, because staining studies with these cells indicate that they display a lower but substantial level of HLA-A2 on their surface (data not shown). The addition of 3.2G1 Ab to the cells resulted in CDC that titrated with the ratio of GVL to TMT. Lysis was not seen for nonpulsed cells (the value was below the spontaneous release in the absence of Ab) or those pulsed with TMT (CDC/H11005 2%). This experiment implies that the degree of lysis reflects the Ag density on the cell.

In a second experiment, an examination of the relationship between target density and cell lysis was conducted using T2 cells that were pulsed with varying levels of GVL peptide alone (Fig. 2B). The peptide was serially diluted and added to cells at concentrations ranging from 100 to 0.1 μg/ml. VLQ peptide and non-pulsed cells were used as a zero-point control. After pulsing and addition of Ab at 10 μg/ml, cells were subjected to CDC analysis. The HLA-A2-specific lysis in the presence of BB7.2 varied from 55 to 85%, whereas that driven by 3.2G1 varied from 10 to 60% (Fig. 2B), titrating with the dose of peptide used to pulse cells. Although there was no indication of a significant decrease in cell lysis for BB7.2, there was a clear relationship between target density and cell lysis demonstrated by 3.2G1, with half-maximal lysis occurring at a peptide concentration of around 6 μg/ml.

In the final CDC experiment involving T2 cells, we further examined the specificity of lysis by the Ab using HLA-A2-peptide tetramers to compete for 3.2G1 binding. 3.2G1 TCRm was serially diluted and preincubated with tetramer such that the final concentrations of TCRm varied from 9 to 0.1 μg/ml and the tetramer concentration was 2 μg/ml after addition to the CDC reaction. Tetramers refolded with the GVL peptide (competitor) substantially inhibited CDC, whereas those refolded in the presence of VLQ peptide (noncompetitor) resulted in an Ab lysis profile almost identical with that seen with no tetramer (Fig. 2C). Taken together, these findings support the fine recognition specificity of the 3.2G1 TCRm mAb for targeting the GVL-A2 epitope on T2 cells for cell lysis by CDC.

3.2G1 detects endogenous GVL peptide-HLA-A2 presented on human tumor cell lines

The ability of the 3.2G1 Ab to detect endogenously processed peptide in the context of the HLA-A2 molecule was evaluated by immunofluorescent staining of a series of tumor cell lines (Fig. 3).
BB7.2 mAb indicated the level of HLA-A2 expression on cells. SKOV3.A2 and SW620 are ovarian and colon cancer cell lines, respectively, whereas MDA-MB-231 and BT20 are breast cancer cell lines. Additional analysis of the SKOV3.A2, SW620, and MDA-MB-231 cell lines by ELISA indicated that hCG was present in these lines (data not shown). BT20 cells were not evaluated for the presence of hCG but were included as an HLA-A2-negative control. The three HLA-A2-positive tumor cell lines displayed different levels of GVL/A2 when stained with the 3.2G1 TCRm, and as might be anticipated, the staining intensity varied in accordance with the level of HLA-A2 on the surface. The HLA-A2-negative cell line, BT-20, was not stained with either 3.2G1 or BB7.2. Because of its consistently high level of expression of GVL/A2 and to maximize the target density, we selected the MDA-MB-231 cell line as the target for the following in vitro and in vivo assays.

The 3.2G1 TCRm mAb directs killing of a human tumor cell line in vitro

The breast cancer cell line MDA-MB-231 was subjected to competition analysis via tetramer blockade of CDC in the same manner in which the T2 cells were evaluated (described above). Cells were plated and allowed to adhere overnight before Ab or Ab plus tetramer was applied. Ab concentration was varied from 25 to 1 μg/ml and tetramer concentration was held constant at 6 μg/ml. CDC of cells incubated with Ab in the absence of tetramer showed an Ab concentration-dependent lysis which was paralleled by cells incubated with Ab in the presence of VLQ tetramer. This indicated that there was essentially no competition provided by the tetramer (Fig. 4A). In contrast, cells incubated in the presence of Ab plus GVL tetramer were almost completely protected from lysis even at the highest concentration of Ab used. These findings further demonstrate the specificity of the 3.2G1 TCRm and indicate that use of this class of Ab as a full-length molecule offers a novel approach for targeting and killing tumor cells.

A second mechanism that plays an important role in the ability of a therapeutic Ab to control or eliminate tumors is ADCC (12, 13, 15). To investigate the ability of the 3.2G1 TCRm mAb to direct ADCC, we isolated PBMC from the platelet chambers of apheresis collection devices from anonymous donors. The cells were held in serum-free medium (AIM-V) containing 200 U/ml recombinant human IL-2 for 2–7 days, with medium changes every 2–3 days to maintain and activate the NK population (25). To determine the level of NK activity present in the different donor samples, each preparation was evaluated using the NK-sensitive cell line K562 at the same time the ADCC assays were conducted. All PBMC isolates were shown to exhibit lysis levels >60%, with one exception (35%) (data not shown).

MDA-MB-231 cells were first evaluated for sensitivity to ADCC as adherent cultures using five different human PBMC preparations to control for variation among the individual donors. Fig. 4B shows the results of these assays, which contained 10 μg/ml 3.2G1 TCRm and were run at an E:T ratio of 30:1. The PBMC preparations varied in their ability to lyse MDA cells as might be anticipated due to differences in receptor expression by NK cells. The overall ADCC ranged from 6.8 to 9.6%, with an average value of 8.7%.
isotype-treated group had developed tumors that were the last tumor in the control mice. At day 69, 8 of 10 mice in the scoring was tabulated on day 69, 21 days after the appearance of and expand in the control mice until day 49 (week 6 tumor 43 days (week 6) after implantation, while none were evident in injection of either 3.2G1 TCRm or an isotype control Ab. Tumors Ab treatment was initiated at the time of implantation with an i.p.

To establish the ability of the 3.2G1 TCRm to inhibit tumor growth MDAMB-231

In vivo analysis of 3.2G1 TCRm in nude mice implanted with MDA-MB-231

To determine the effect epitope density had on overall lysis, 3.2G1 TCRm or the pan-HLA Ab W6/32, which is also a murine isotype IgG2a, were used as targeting agents. Fig. 4C shows the results from an ADCC analysis of MDA-231 cells using two different human donor preparations at an E:T ratio of 20:1 with 3.2G1 and W6/32. The lysis values achieved for W6/32 (14.6–22.6%) were greater than those of 3.2G1 (6.4–13.4%), suggesting that lysis was at least in part dependent on epitope density. Overall, these results show a modest but consistent level of tumor-specific ADCC mediated by the 3.2G1 TCRm.

In vivo analysis of 3.2G1 TCRm in nude mice implanted with MDA-MB-231

To establish the ability of the 3.2G1 TCRm to inhibit tumor growth in vivo, we implanted nude mice with MDA-MB-231 tumor cells. Ab treatment was initiated at the time of implantation with an i.p. injection of either 3.2G1 TCRm or an isotype control Ab. Tumors began to appear in the isotype control-treated mice between 36 and 43 days (week 6) after implantation, while none were evident in any of the mice treated with 3.2G1. Tumors continued to appear and expand in the control mice until day 49 (week 6 tumor volume = 4.5 mm3; week 10 tumor volume = 157 mm3). Final scoring was tabulated on day 69, 21 days after the appearance of the last tumor in the control mice. At day 69, 8 of 10 mice in the isotype-treated group had developed tumors that were ≥6 mm in diameter, while none of the nine mice in the group treated with the 3.2G1 TCRm showed evidence of tumor growth (Fig. 5). The experiment was terminated at 71 days.

Discussion

The current study characterizes the functional properties of an Ab with the type of HLA-restricted peptide specificity associated with TCR. The similarity in epitope recognition to a TCR has led us to designate this Ab a TCRm and to investigate its potential as a therapeutic agent. The 3.2G1 TCRm is a murine IgG2a mAb that 1) can mediate the staining and lysis of cells bearing the GVL peptide-HLA complex on their surface by both CDC and ADCC. The therapeutic utility of the bacteriophage-derived Fab Abs have been investigated by creating genetic constructs in which the Ab’s recognition domain is used to specifically target cells expressing the peptide-HLA complex. Targeting of influenza-infected cells or melanoma cell lines with anti-peptide-MHC Fab-immunotoxin conjugates has been shown to inhibit cell growth in vitro (35, 36). One of the limitations of using phage display is that the random association of L and H chain fragments may not generate binding pairs of the highest affinity. For example, Chames et al. (37) subjected a phage-derived Fab to mutagenesis to develop a 2-log increase in staining sensitivity and more efficient tumor killing by the MAGE/A1-specific TCRm. The advantages of developing TCR mimic IgG mAbs include the acquisition of matched heavy and L chain pairs that have been affinity matured in the animal and the ability to activate immune effector functions via the Fc domain of the molecule without further genetic modification. In vitro and in vivo characterization of full-length Ab-like molecules that recognize an MHC-restricted peptide are rare. Aharoni et al. (34) reported the use of an Ab, made by the hybridoma fusion technique, to a murine MHC-II molecule loaded with a basic protein from rat in a CDC assay format. Mosquera et al. (38) have reported the in vitro and in vivo characterization of a TCR-human IgG chimeric fusion protein that is specific for the p53 peptide (264–272). In this report, we showed the efficacy of the 3.2G1 TCRm as a therapeutic agent by examining its ability to trigger CDC and ADCC of a tumor line in vitro and prevent tumor growth in vivo. To our knowledge, this is the first demonstration of use of a “naked” TCRm mAb as a therapeutic agent.

Elimination of tumors in vivo by Ab therapy is thought to be the result of any or all of a number of mechanisms including blockade of growth factor receptors, induction of apoptosis, CDC and ADCC. The efficacy of growth factor receptor blockade has been shown in tumor xenotransplantation experiments with murine
anti-EGF and anti-neu Abs which significantly inhibit tumor growth in mice (16, 39). These effects appeared to be independent of Ab isotype because IgG1, IgG2a, and IgG2b suppress growth, whereas only the IgG2a and 2b isotypes show any in vitro activity in CDC and ADCC experiments. In contrast with Abs that bind and block growth factor receptors, studies using Abs that are directed at other targets, such as Abs that recognize surface IgM or Thy 1.1 on murine lymphomas, show a direct correlation between in vitro activity and tumor regression (40, 41). Studies by Clynes et al. (42) using Fcγ/H9253RII-chain knockout mice demonstrated that the efficacy of anti-HER2 and anti-CD20 Abs (trastuzumab and its murine Ab predecessors and rituximab) is significantly reduced in the absence of functional receptors. In addition, they demonstrated that inactivation of the inhibitory Fcγ/H9253RII increased ADCC, indicating that FcR-mediated events play an important role in tumor suppression (15). Mice deficient in the initiating factor for the classical complement pathway, C1q, were used to show that this pathway is essential for the therapeutic activity of rituximab (43).

CDC of peptide-pulsed TAP-deficient T2 cells is efficiently driven by the 3.2G1 TCRm in our study. Although CDC has been used for years for tissue typing and was reported for an Ab specific for a peptide-MHC-II complex of murine origin (31), it was not clear that recognition of peptide-HLA epitope would result in the efficient promotion of cell lysis. In addition, they demonstrated that inactivation of the inhibitory FcγRII increased ADCC, indicating that FcR-mediated events play an important role in tumor suppression (15). Mice deficient in the initiating factor for the classical complement pathway, C1q, were used to show that this pathway is essential for the therapeutic activity of rituximab (43).

Comparison of the results of CDC analysis of peptide-pulsed T2 cells and the MDA-MB-231 tumor line appeared to demonstrate that a higher Ab concentration was necessary to achieve lysis and that overall lysis was lower for the MDA-MB-231 tumor line. A number of known inhibitors of the complement system exist, including CD46, CD55, and CD59 (membrane cofactor of proteolysis, decay-accelerating factor, and protection or Mac inhibitor, respectively). Although the exact effect of these inhibitors on the complement components present in the rabbit serum used in these studies is disputed, it follows that their presence may reduce or inhibit lysis (44–47). MDA-MB-231 cells have been reported to express moderate levels of CD46 and high levels of CD55 and CD59 when evaluated by flow cytometry (13). Samples from patients with multiple myeloma and non-Hodgkins lymphoma (NHL), as well as NHL cell lines that were resistant to CDC, tested positive for these markers (23, 48). Blockade of the inhibitors enhanced lysis in all cases with CD59 neutralization having the most dramatic effect (23). Thus, it appears that reduced lysis of the MDA cells in our experiments could be linked to the presence of specific inhibitors of CDC expressed by this line.
The ADCC assays presented indicated that the two murine Abs that recognize human HLA, 3.2G1, and W6/32, were at best moderately efficient at directing lysis by NK cells. These results do not differ significantly from those reported for rituximab by Harjunpaa et al. (23) or Golay et al. (24), in which various NHL cell lines were tested and the level of cytotoxicity was reported to be between 8 and 25% specific lysis. Harjunpaa et al. (23) categorized this level of lysis as relatively ineffective, whereas Golay et al. (24) concluded that both CDC and ADCC are major mechanisms of action on B cell lymphomas. The issue of target density and threshold level was addressed here by inclusion of mouse Ab W6/32. This Ab recognizes a conformational motif on β2m, is of the same isotype, and displays ~5-fold increased MFI when used in flow cytometric analysis. However, it does not increase the specific lysis >2-fold in the most active IL-2-stimulated human PBMC preparation. The existence of inhibitory receptors on NK cells and their engagement by HLA or other ligands on the target cell surface has been shown to be a powerful inhibitor of NK lytic signaling. HLA-C is thought to be the most dominant class I molecule involved in inhibitory signaling and MDA-MB-231 cells express two HLA-Cw4 alleles (CD158b and b2) known to be recognized by the inhibitory receptor KIR2DL2/3, which is present on most NK cells (49–52). Palmieri et al. (53) have shown that simultaneous stimulation of another inhibitory receptor, CD94/NKG2A, and FcγRIII (CD16) substantially inhibits signaling in NK cells related to cytolytic activity. Multiple myeloma and NHL tumor lines as well as patient samples resistant to rituximab were found to express proteins that inhibited ADCC (48, 54). These proteins are also expressed by MDA-MB-231 cells, which may explain their resistance to both CDC and ADCC. The cumulative effect of these inhibitory mechanisms appears to lead to suppression of NK-mediated lysis in our assays. However, it remains to be resolved how best to reconcile these results with those we obtained in vivo with nude mice. Despite low levels of in vitro cytotoxicity, our in vivo studies suggest that the murine innate response was triggered to a level that led to complete protection from tumor implantation and growth. The elucidation of the contributions of the specific receptors which affect these two pathways offer interesting possibilities for further research and the potential for therapeutic intervention.

The results obtained with our novel TCRm indicate that 1) the peptide-MHC complex is a legitimate target for cancer therapy by a naked Ab, 2) the level of expression of specific complex is high enough on at least one tumor line to lead to efficient lysis, and 3) there appears to be a threshold level of expression of the complex above which the Ab is effective. A large number of peptide Ags from tumors that are recognized by T cells have been characterized previously (55) and now offer new targets available on the tumor surface for Ab therapy. These Abs open access to a new range of targets available on the cell surface that are independent of the ultimate location of the original protein to which they are directed. The ability to create effective TCRm recognizing such peptides in the context of MHC Ags presents the opportunity to significantly expand the current repertoire of therapeutic Abs.

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Disclosures
J. A. Weidanz is chief scientist and founder of Receptor Logic, Ltd.

References


