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The in vitro biological activity of the HLA-DR-binding clinical IgG4 antibody 1D09C3 is a consequence of the disruption of cell aggregates and can be abrogated by Fab arm exchange

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ABSTRACT

Antibodies of the IgG4 subclass, directed against cell surface antigens have received attention as therapeutic molecules due to their poor induction of the complement system. The MHC class II-directed IgG4 antibody 1D09C3 has been explored for the treatment of lymphomas. The mechanism-of-action is still controversial. Apoptosis induction following HLA-DR engagement has been proposed. However, the validity of these results has been questioned by the observation that antibodies may induce formation of cell aggregates and cell death is induced upon dispersion of these aggregates prior to the quantification of cell death by flow cytometry. Here we address the capacity of 1D09C3 to induce apoptosis in vitro, also taking account of the recently reported Fab arm exchange of IgG4 antibodies. 1D09C3 induces formation of tight cellular aggregates that can only be dispersed at the expense of massive cell damage and death. Using dual color fluorescence cross-correlation spectroscopy (FCCS) we demonstrate that also this antibody undergoes Fab arm exchange in the presence of IgG4. FCCS is a powerful technique to investigate the molecular mechanism of Fab arm exchange using minute amounts of reagents. Following exchange, the functionally monovalent 1D09C3 chimeras loose their ability to induce aggregate formation of HLA-DR-positive cells. Neither functionally monovalent nor bivalent 1D09C3 antibodies induce cell death or apoptosis in myeloma target cells, when microscopy instead of flow cytometry is employed as the analytical technique. Our results indicate that the activity of 1D09C3 in vitro may have been a consequence of assay design rather than an ability to induce HLA-DR-dependent cell death.

1. Introduction

Induction of tumor cell death by targeting tumor cell surface antigens with monoclonal antibodies has brought about a major progress in cancer therapy, as shown by the success of Rituximab, directed against CD20, and alemtuzumab recognizing CD52, in the treatment of lymphomas (Castillo et al., 2008; Dalle et al., 2008; Fanale and Younes, 2007). These antibodies exert their effect by recruiting the complement system (CDC) and/or inducing antibody-dependent cell-mediated cytotoxicity (ADCC).

The human leukocyte antigen (HLA)-DR is one of the three highly polymorphic class II major histocompatibility complex molecules, expressed on cells of the immune system and more specifically B-cells, activated T-cells, monocytes and dendritic cells (Kaufman et al., 1984). In contrast, other somatic cells do not express HLA-DR, or express it only at low levels, which facilitates targeting of malignant tumor lymphoma cells expressing this surface marker. Preclinical evidence suggested that B- and T-cell tumors can be killed by the engagement of receptors involved in activation and growth (Ashwell et al., 1987; Vuist et al., 1994). Cell killing upon MHC II engagement has been reported for the murine system (Bridges et al., 1987) and for human cells in vitro (Nagy et al., 2002).

Several mechanisms have been implicated in the induction of cell death by anti-HLA-DR antibodies. In addition to ADCC and CDC, there have been reports on sensitization towards CD95/Fas-dependent apoptosis (Truman et al., 1997; Yoshino et al., 1995) as well as direct induction of apoptosis (Truman et al., 1994), the latter being either caspase dependent (Blancheteau et al., 2002) or independent (Mone et al., 2004; Nagy and Mooney, 2003).

The fully human anti-HLA-DR antibody termed 1D09C3 originated from screening the Human Combinatorial Antibody Library Hu-CAL (Nagy et al., 2002) and was engineered as an IgG4 antibody. IgG4 antibodies differ functionally from other IgG subclasses

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by their poor ability to induce the complement system thus minimizing side effects due to Fc-portion-mediated effector functions. In vitro experiments have shown a strong tumoricidal effect and activity on a series of lymphoma cell lines (Nagy et al., 2002). In vivo, the treatment of SCID mice inoculated with Granta-519 cells, derived from mantle cell lymphoma (MCL) resulted in a significant delay in tumor growth. 1D09C3 was shown to induce mitochondrial membrane depolarization and production of reactive oxygen species but not caspase activation (Carlo-Stella et al., 2006).

For the major part, these results on 1D09C3-induced apoptosis were based on flow cytometry-based cell viability assays. The validity of such assays has recently been questioned (Mattes, 2007). For the monoclonal antibody L243, that also binds HLA-DR, the author noted that antibody binding can induce extensive cell aggregation. Dispersing the aggregates by harsh measures not only released single cells from the clusters but also caused massive cell death. This result warrants a critical evaluation of in vitro results obtained for 1D09C3.

Another relevant feature of antibodies of the IgG4 subtype is the exchange of Fab arms. As a consequence, blood-derived IgG4 molecules are unable to crosslink antigens, which has been referred to as functional monovalency (van der Zee et al., 1986). A recent report has validated the model that antibody fragments can be exchanged between IgG4 molecules, thereby creating chimeric immunoglobulins with specificity for two different antigens (van der Neut Kolfschoten et al., 2007). For this phenomenon the terms Fab arm exchange and Fab fragment swapping were coined, even though one light and one heavy chain, rather than Fab arms are exchanged in this rearrangement. The exchange depended on a low molecular weight compound present in erythrocyte lysate that was suggested to be identical to reduced glutathion. The effect was shown in vitro as well as in vivo.

Here we addressed whether 1D09C3 would also undergo Fab arm exchange and what the functional consequences of this exchange would be. Until now, the biological activity of 1D09C3 was only investigated in SCID mice, in which such exchange cannot occur. In contrast, in humans, Fab arm exchange could result in functional monovalency.

Thus far, rather cumbersome ELISA assays were employed for the detection and quantification of Fab arm exchange. Provided that the molecule of interest can be fluorescently labeled, confocal fluorescence correlation spectroscopy (FCS) (Rigler et al., 1993) and more specifically fluorescence cross-correlation spectroscopy (FCCS) can deliver quantitative information on molecular interactions at nanomolar concentrations of probe molecules with very small sample volumes (Schwille et al., 1997). In FCCS, both interaction partners are labeled with fluorophores that do not spectrally interact. This prerequisite sets the method apart from fluorescence resonance energy transfer (FRET) (Clegg, 1995). Typically, a fluorescein-like dye as for example Alexa Fluor 488, emitting fluorescence around 520 nm is combined with a Cy5-like dye such as Alexa Fluor 647 emitting fluorescence around 670 nm. For this dye combination, two laser lines of the appropriate wavelengths for excitation are focused into the sample. The fluorescence of either fluorophore is detected by a separate detector, using confocal optics to confine the detection volume to the size of an *Escherichia coli* bacterium along the optical axis. Molecules carrying both fluorophores and diffusing through the detection volume reveal themselves by a concerted fluctuation of fluorescence in both channels.

Using FCCS, flow cytometry and confocal fluorescence microscopy, we address the capacity of 1D09C3 to undergo Fab arm exchange and to induce cell death in vitro both in its bivalent as well as in its functionally monovalent form.

2. Materials and methods

2.1. Cell lines

The human-derived tumor cell line Priess, a human B-lymphoblastoid line, was originally obtained from the European Collection of Cell Cultures, Center for Applied Microbiology & Research (Salisbury, UK). The human Hodgkin lymphoma cell line KM-H2, B-cell non-Hodgkin lymphoma line Granta-519, and the human multiple myeloma cell line OPM-2 were purchased from the DSMZ (Braunschweig, Germany). High expression levels of HLA-DR were reported for Priess, Granta-519 and KM-H2 cells, while HLA-DR is not expressed in OPM-2 cells (Nagy et al., 2002).

Cells were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) containing 10% fetal bovine serum (FBS, PAN Biotech, Aidenbach, Germany) at 37 °C and 5% CO_2 and were routinely tested for mycoplasma infection (MycoAlert Mycoplasma Detection Kit, Cambrex Bio Science, Nottingham, UK).

2.2. Antibody labeling

Labeling of the antibodies using Alexa Fluor 488 and Alexa Fluor 647 succinimidyl esters (Invitrogen) and the subsequent purification were performed as described in the manufacturer's manuals. Briefly, antibody stock solutions of about 2 mg/ml for 1D09C3 (EBEWE Pharma, Unterach, Austria) and 1.2 mg/ml of human myeloma IgG4 (Sigma, Germany) were prepared in PBS plus sodium bicarbonate (pH 8.3). An aliquot of either antibody solution was added to a separate vial containing the amine-reactive dye and a magnetic bar and stirred for 1 h at room temperature.

Purification was accomplished on a gravity-driven size-exclusion column. The purification columns were assembled and filled with purification resin as described in the manual. The antibody dye mixtures were loaded onto the column, rinsed and eluted in PBS (PAA Laboratories GmbH, Linz, Austria), pH 7.2, containing 0.2 mM NaN_3. The first colored band containing the labeled protein was collected. The labeling efficiency was determined by UV/Vis spectroscopy following the manufacturer's guidelines. Dye/antibody ratios were as follows: Alexa Fluor 488-1D09C3: 5.3, Alexa Fluor 647-1D09C3: 6.7, Alexa Fluor 488-IgG4: 3.5, Alexa Fluor 647-IgG4: 13.

2.3. Fluorescence cross-correlation spectroscopy (FCCS)

FCCS measurements were performed in 384-well plates (175 μm, low-base design, MMI, Eching, Germany) using a ConfoCor 2 fluorescence correlation spectroscope equipped with a C-Apochromat 40× N.A. 1.2 water immersion lens (Carl Zeiss, Jena, Germany) and a TCS SP5 confocal laser scanning microscope equipped with a dual channel fluorescence correlation spectroscope and an HCX PL APO 63× N.A. 1.2 water immersion lens (Leica Microsystems, Mannheim, Germany). Fluorescence fluctuations were recorded for 10 s with 5 repetitions and afterwards analyzed using the software provided with either microscope. For obtaining the fraction of dual-labeled molecules from the ratio of the auto- and cross-correlation functions (Baudendistel et al., 2005), either FCCS set-up was first calibrated using a synthetic double-stranded oligonucleotide labeled on the 5′-end of either strand with either rhodamine green or Cy5. The 488 nm laser line of an argon-ion laser and the light of a 633 nm helium–neon laser were used for excitation. On the ConfoCor2, this light was directed over an HFT 488/633 and NFT 610 beam splitter and fluorescence was detected after passing the emission filters BP 500-550IR and LP650. On the TCS SP5, a BP 500/550 in combination with a BP 647/703 and a beam splitter BS 625 were employed for the detection of fluorescence. Confocal laser scanning microscopy was performed on