Lonely killers

Effector cell- and complement-independent non-proapoptotic cytotoxic antibodies inducing membrane lesions

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Key words: cytotoxicity, therapeutic antibody, necrosis, membrane lesion, cell death

The majority of the most effective monoclonal antibodies (mAbs) currently in the clinics bind to cancer or immune cells. Classic mechanisms of cell killing by therapeutic mAbs include antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity and induction of apoptosis by engagement of specific cell ligands. A few reports have described mAbs whose cytotoxic activity is Fc-independent and that do not induce the morphological and biochemical changes associated with the apoptosis-type of cell death. Even fewer works describe mAbs able to directly induce membrane lesions. Here, we discuss the available data on those molecules and their cell killing activity, with particular attention to the case of a mAb specific for the tumor-associated N-glycolyl (Neu5Gc)-GM3 ganglioside [GM3(Neu5Gc)]. Some similarities are found in the cell death pathways triggered by these mAbs, but data are not abundant. We conclude that the usefulness of mAbs with a direct cytotoxic activity for immunotherapeutic strategies deserves deeper research.

Targeting Cells with Therapeutic Antibodies

Substantial research efforts are currently devoted to elucidating the mechanisms of action of therapeutic antibodies. These molecules are leading products of the biopharmaceutical industry worldwide, both from the clinical and the market points of view. Although one approved monoclonal antibody (mAb) is directed against an infectious agent (anti-respiratory syncytial virus palivizumab), the majority of both approved and pipeline mAbs are evaluated as treatments for chronic non-transmissible diseases, particularly cancer.

In the field of tumor immunotherapy, mAbs are well ahead of cancer vaccines in terms of clinical efficacy and approval by regulatory agencies. Cancer is a very complex and diverse pathology. To date, it has been postulated that a normal cell may acquire at least ten capabilities in becoming a “successful” tumor: sustained proliferative signaling; the evasion of growth suppressors; avoidance of an immune response; the possibility of replicative immortality; induction of tumor-promoting inflammation; invasiveness and metastatic potential; induction of neoangiogenesis; genome instability with accumulation of mutations; insensitivity to normal cell death pathways; and deregulation of the energetic metabolism. Each of these steps is susceptible to different therapeutic strategies, which increasingly includes the use of mAbs either as single agents or in combination with other cancer drugs.

Approved anti-cancer mAbs target not only tumor-associated antigens, but also molecules important for the tumor micro-environment or displayed by immune cells, e.g., anti-CD20 rituximab (Rituxan®), anti-CD52 alemtuzumab (Campath®), anti-HER2 trastuzumab (Herceptin®), and anti-epidermal growth factor receptor (EGFR) cetuximab (Erbitux®) and nimotuzumab (CIMAher), which belong to the first group; anti-vascular endothelial growth factor (VEGF) bevacizumab (Avastin®), and anti-CTLA4 ipilimumab (Yervoy®), from the second and third groups, respectively. An alternative approach to cancer immunotherapy is the use of anti-idiotypic vaccines. In this case, the antibodies generated against the immunoglobulin acting as immunogen are supposed to recognize the tumor-associated antigen. Although understanding the carcinogenesis process and its interaction with the immune system is leading to more effective and combined treatments, currently the majority of the mAbs with clinical efficacy directly target tumor cells. Bound mAbs can then trigger a number of cell death mechanisms that may or may not involve immune effectors.

Most therapeutic mAbs against autoimmune diseases neutralize soluble and membrane-bound proinflammatory cytokines, e.g., anti-tumor necrosis factor (TNF) infliximab (Remicade®) and adalimumab (Humira®), which can also induce cell death. Other mAbs target surface molecules on immune cells, e.g., rituximab and alemtuzumab, which exert cytotoxicity by different mechanisms over B lymphocytes; and anti-CD3 otelixizumab (TRX4), teplizumab (MGA031), ior t3, and anti-CD6 itolizumab (T1hT), which all modulate T-cell function.
In this review, we analyze the published data on non-apoptotic mAbs still able to kill target cells without the intervention of cytotoxic cells or complement. In particular, we focus on mAbs that cause cell death by affecting membrane integrity, particularly an antibody specific for the tumor-associated N-glycolyl (Neu5Gc)-GM3 ganglioside [GM3(Neu5Gc)].

Considerations on “Classic” Antibody-Mediated Cell Death Mechanisms

Antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and apoptosis are currently regarded as the main effector functions of therapeutic mAbs for the killing of target cells. For instance, from the above mentioned mAbs that bind to cells, rituximab is known to exert all of them.

Although still the subject of some controversy, methods to measure and characterize the different types of cell death are being unified. It is of paramount importance to assign to each cell killing activity the correct classification, thus contributing to understand the mechanism of action of therapeutic mAbs. Probably the most popular current technique is cytfluorimetry, which is widely used to quantify the above described mechanisms. Interestingly, a cytfluorimetric assay was recently proposed to differentiate ADCC from antibody-dependent cell-mediated phagocytosis (ADCP). Briefly, target cells stained with a vital dye and incorporating another non-vital one are considered to undergo cytotoxic killing, while those being phagocytized are identified by staining the phagocytic cell with a specific antibody-fluorophore conjugate. Using this assay, these authors postulated that the ADCC activity assigned to known therapeutic mAbs may be overestimated, while the ADCP is normally not determined. In fact, they demonstrated that when trastuzumab isotype (gamma1) is switched to epsilon (thus obtaining a therapeutic IgE), the relative contribution of ADCC and ADCP to the cell killing properties of the IgG1 and the IgE mAbs is evidenced. Surprisingly, the IgE antibody mediated more ADCC than the IgG1, while the latter was more efficient in ADCP. The potential use of therapeutic IgE to treat cancer, termed allergen-therapy, is an emerging field with interesting preliminary results.

Another polemic issue is the unequivocal definition of the apoptosis-type of cell death. Apoptosis is often considered equivalent to death with caspase activation, but today it is well-documented that this is not always true. There are several methods for identifying apoptosis, the most frequently used of which includes nevertheless caspase activation, as well as the activation of proapoptotic proteins, DNA fragmentation and phosphatidylserine exposure, among others. On the other hand, the necrosis-type of cell death, which is sometimes considered as an unregulated process despite evidence that it can occur through well-controlled pathways, is defined by exclusion criteria, i.e., neither apoptosis nor cell dying with autophagy. The fourth type of cell death is cornification, which is a protective mechanism exclusive of the skin. Therefore, it can easily be inferred that the mAbs to whom the title of this review refers to, induce necrosis on cancer cells. As mentioned earlier, tumors “evolve” to avoid destruction by the immune system, and the killing activities of therapeutic mAbs are also influenced by different patient factors. ADCC and CDC effectiveness is modulated by the host polymorphism in the Fcy receptors (FcγRs) and key components of complement, such as C1q, respectively. CDC can also be impaired through the expression by the tumor of complement inhibitor surface proteins. Apoptosis is susceptible to alteration as a consequence of the modification of several cellular pathways. However, the most obvious way to circumvent the antibody attack is the change in antigen density or distribution in the malignant cell. It has thus been suggested that a successful strategy for cancer immunotherapy should be based in the simultaneous targeting of at least two independent molecules.

Active effects of passive antibodies. The study of the mechanisms of action of therapeutic mAbs targeting cells directly have recently gone beyond their passive effects, i.e., tumor burden reduction by direct killing of cells through ADCC, CDC or apoptosis. This has come from the observation of long-lasting immune responses after the termination of mAb administration, which suggests the activation of endogenous responses.

The vaccinal effect of mAbs has been well documented for rituximab. A recent example is that of 7A7, a mAb used as preclinical model of nimotuzumab. Besides inducing ADCC, administration of 7A7 activated an anti-tumoral T-cell response. It was later demonstrated that this antibody induces an immunogenic apoptosis-type of cell death. Although apoptosis is generally considered to be silent in terms of immune system activation, under certain conditions, such as delayed phagocytosis of apoptotic bodies, apoptotic cells can provide signals able to prime adaptive immune responses. Therefore, the relative contribution of each type of cell death for the vaccinal effect of therapeutic mAbs remains controversial because necrotic cells can also be poorly immunogenic or even have tumor-promoting effects.

Cytotoxic Antibodies Affecting Membrane Integrity

Antibodies with the ability to kill cells by themselves, in a non-apoptotic way, are rarely described in literature. BR96 is a mAb specific for Lewis y (Le’) that has been used as immunotoxin in clinical trials of patients with carcinomas expressing this antigen. The antibody was demonstrated to induce in vitro a direct cytotoxic activity that involved loss of membrane integrity.

In 1995, a novel mechanism of cell killing by an antibody, which did not meet apoptosis criteria, was described. The antibody, RE2, was obtained by immunizing rats with a lysate of mouse T cells. The most striking finding was the induction on activated mouse T cells of extensive lesions (described by the authors as “giant holes”) that were perfectly visible by scanning electron microscopy.

A group of mAbs recognizing lymphocytes, but in this case human B cells, were reported to have a similar cytotoxic effect. Cells incubated with 216 mAb and similar human IgMs, which bind to a surface carbohydrate similar to “i” antigen on cord
Table 1. Features of non-proapoptotic membrane lesion-inducing cytotoxic mAbs

| mAb        | Species, isotype | Antigen                                      | Fab activity | Caspase activation | Apoptotic DNA fragmentation | Cytoskeletal changes | Cytotoxicity at 4°C | Energy dependence | Ca" influx | Refs. |
|------------|------------------|----------------------------------------------|--------------|--------------------|-----------------------------|----------------------|-------------------|-------------------|-------------|-------|
| BR96       | Mouse IgG        | Lewis y                                      | ND           | ND                 | ND                          | +                    | ND                | ND                | ND          | 38, 42 |
| RE2        | Mouse IgG        | MHC class I α2 domain (cytotoxicity dependent on T-cell activation) | -            | -                  | -                           | +                    | -                 | -                 | -           | 43, 50 |
| 216 and other | Human IgMs      | Surface carbohydrate on human B cells similar to “i” antigen on cord erythrocytes | ND           | ND                 | ND                          | +                    | ND                | -                 | -           | 44, 45 |
| Anti-Porimin | Mouse IgM       | Carbohydrate epitope on Porimin (mucin)       | ND           | ND                 | ND                          | ND                   | ND                | ND                | ND          | 46, 47 |
| KID3 / RAV12 | Mouse IgG / Chimeric IgG | N-linked carbohydrate RAAG12 antigen | -            | ND                 | ND                          | +                    | ND                | ND                | ND          | 48    |
| 84         | Mouse IgM        | podocalyxin-like protein-1                   | ND           | -                  | -                           | +                    | -                 | ND                | ND          | 51, 52 |
| 14F7       | Mouse IgG (also chimeric and humanized IgG1) | GM3(Neu5Gc) ganglioside | -            | -                  | -                           | +                    | +                 | -                 | -           | 65, 66, 68 |

ND, not determined.

erythrocytes, exhibited large membrane lesions (or “pores,” according to these authors).44,45 A mouse IgM specific for a surface receptor on human Jurkat T cells, named anti-Porimin (derived from the phrase ‘pro-oncosis receptor inducing membrane injury’), also induced direct cell death with the formation of membrane pores.46 Cells transfected with this receptor were also killed by this antibody with cell membrane injury.47

RAV12 is a chimeric antibody that recognizes the N-linked carbohydrate antigen RAAG12.48,49 It was constructed from the mouse mAb KID3. Both antibody versions induced membrane rupture upon binding to a colon tumor cell line.48 RAV12 was evaluated in a Phase 1 clinical study of patients with recurrent adenocarcinoma.48

An IgM mAb that recognizes human embryonic stem cells, specifically through the podocalyxin-like protein-1, was found to be directly cytotoxic to these cells.31 The 84 mAb induced membrane pores in treated cells.52 Another well characterized antibody exhibiting this effect on plasma membrane is 14F7 mAb,53 which targets the tumor-associated GM3(Neu5Gc).14

14F7 mAb. Neu5Gc-sialoconjugates are absent from human normal tissues, but tumors can differentially incorporate them from exogenous sources;14 thus, they become like tumor-specific antigens. GM3(Neu5Gc) is a ganglioside whose expression has been detected in some human tumors, including breast and melanoma.53,55,56 Several therapeutic strategies have been developed against this target,14 e.g., vaccines (ganglioside-based57 and anti-idiotypic58-63) and mAbs.55 14F7 mAb is specific for this ganglioside and unable to bind its N-acetylated (Neu5Ac) counterpart.53 Its exquisite specificity for GM3(Neu5Gc), which differs from GM3(Neu5Ac) only in the presence of a hydroxyl group instead of a hydrogen atom, has been explored with structural studies.64 14F7 mAb was able to recognize breast and melanoma tumors by immunohistochemistry,53 and also breast cancer by radioimmunoscintigraphy, in a Phase 1/2 diagnostic clinical study.55

14F7 mAb displayed anti-tumor properties in vivo against a GM3(Neu5Gc)-expressing murine myeloma. In vitro, the antibody was shown to induce both ADCC and CDC, but the most interesting finding was a direct cytotoxic activity over the cells.65 Cells treated with 14F7 mAb showed big “holes” on their plasma membrane,66 similar to those reported previously for RE2,43 216 44,45 and 84 52 mAbs. A humanized version of 14F7 mAb, obtained by the modification of potential human T-cell epitopes on the variable region of both antibody chains,67 retained this ability.68 Interestingly, the recombinant antibody-producing cell line was also sensitive to 14F7 mAb-induced cytotoxicity. Therefore, for scaling up the production process, it was necessary to genetically modify these cells in order to impair the synthesis of GM3(Neu5Gc).68

Mechanism of membrane lesion formation. The available data on the molecular mechanism(s) underlying the appearance of these plasma membrane holes or pores triggered by the above described mAbs are scattered and incomplete (Table 1). This cell death pathway, involving cell and organelle swelling, vacuolization, blebbing, membrane rupture and karyolysis (as opposed to apoptotic karyorrhexis), was previously referred to as “oncosis,”69 but is now recognized as an initial step of necrosis.18 A feature
shared by these mAbs is the induction of rapid cell death, often within 5–20 min of incubation. 52-54,56,59

Cytoskeleton reorganization in treated cells has been demonstrated for RE2,70 and 14F7 66 mAbs. Addition of the actin polymerization inhibitor cytochalasin impaired the cytotoxicity induced by these mAbs. Moreover, it was demonstrated that in the RE2 mAb killing mechanism, the LFA-1 integrin plays an important role,59 and 14F7 mAb binding induces phosphorylation of ezrin.66 This protein is part of a triad known as ERM (ezrin-radixin-moesin), which regulates the association between membrane proteins and the cytoskeleton and participates in signal transduction pathways.71 Upon phosphorylation, ezrin links the plasma membrane to actin cytoskeleton, and also interacts with transmembrane proteins. Notably, podocalyxin binds to actin through ezrin,72 and the antigen recognized by 84 mAb is a podocalyxin-like protein.51 In the case of cells treated with 84 mAb52 and KID3/RAV12,48 a disruption of the actin cytoskeleton was observed.

BR96 is an internalizing antibody.38,42 Its cytotoxic activity thus begins with membrane infolding, with its subsequent internalization, cell surface and intracellular vesicle formation and loss of membrane integrity. This process required antigen cross-linking and occurred also at 4°C.42 These two latter features are shared by RE2 70 and 14F7 66 mAbs. 84 mAb was also cytotoxic at 4°C.38 Interestingly, in the case of 216 mAb cytotoxicity levels increased at this temperature when compared with incubation at 37°C.44

Additionally, neither the presence of sodium azide nor of EDTA dampened the killing activity of RE2,70 216 66 and 14F7 66 mAbs, which indicates independence from metabolic energy and calcium influx, respectively. Furthermore, with the respective cycloheximide and actinomycin inhibitors, it was demonstrated that the cytotoxic activity of 14F7 mAb does not require de novo protein or mRNA synthesis.66 For KID3/RAV12, it was demonstrated that sodium influx was necessary for cell killing.48 No data are available for the anti-Porimab mAb,66,67

In summary, there are similarities in the killing mechanism exerted by the above described mAbs (Table 1); nevertheless, the relative contribution for this activity of both the antibody isotype and the nature of the antigen recognized on the target cells is not entirely elucidated.

Affinity vs. cytotoxicity. Although generally a desired feature for therapeutic mAbs, high affinities can also have shortcomings. Cetuximab and nimotuzumab are representative examples of this phenomenon. Both mAbs bind to the EGFR, which is a validated target for cancer immunotherapy.73 Nimotuzumab has a lower affinity,74 and coincidently the adverse effects (mainly a skin rash) it provokes are much less serious compared with those of cetuximab.73-77 Non-mutually exclusive hypotheses have been offered to explain its toxicity profile, including its preferential accumulation in tumor tissues, which have a higher antigen density;75 the need of bivalent binding for exertion of its activity;78 and its unique binding site on the EGFR extracellular domain III.74

Nevertheless, in the case of 14F7 mAb-induced cell death, affinity plays a central role. Evidences of an affinity maturation process were found in 14F7 mAb variable region.70 P3 is a mAb that also recognizes GM3(Neu5Gc), but also other Neu5Gc-gangliosides and sulfatides.80,81 In contrast to 14F7, the chimeric version of P3 82 was unable to kill GM3(Neu5Gc)-expressing cells.66 Originally a germ-line IgM,83 its lower affinity was the explanation given to this observation.66 This was later proved with a more reactive mutated variant of chimeric P3. By replacing a glutamate by an arginine residue at the heavy chain variable region (VH) of P3, thus increasing the number of this residue in the heavy chain complementary determining region 3 (H-CDR3), an antibody able to bind more strongly to P3 mAb glycolipidic ligands, without affecting its interaction with its anti-idiotypic mAbs was obtained.84

Similar observations were made for 216 mAb and its family of human IgMs. These mAbs, which belong to the same V H family, bound a common anti-idiotypic antibody with similar affinities. However, the reactivity to B cells varied, and this positively correlated with the cytotoxicity. Although these are predominantly unmutated IgMs, the strongest binders display basic residues-enriched H-CDR3, with a predominance of arginines.45 The importance of these latter residues for the binding of antibodies to several self-antigens, such as chromatin,85 cardiolipin86 and other phospholipids,87 and gangliosides has been demonstrated.88,89

Unlike the parental antibody, the arginine-enriched mutated P3 variant displayed cytotoxic activity over cells with high expression of the ganglioside. Surprisingly, in contrast to 14F7, this antibody was able to kill cells, although to a lesser extent, devoid of gangliosides but not of Neu5Gc-sialoconjugates in general, suggesting that binding to non-glycolipidic ligands containing this variant of sialic acid can also mediate this effect. Additionally, preliminary results suggested that the mechanism of cell death induced by this mutated P3 antibody might have differences with respect to that of 14F7 mAb, as the characteristic cell swelling observed with the latter was absent in cells treated with the former.84 The possible induction of membrane lesions and the determination of markers of apoptosis are pending.

P3 mAb was used to obtain racotumomab (1E10),90 the anti-idiotypic antibody that is being used as a vaccine for inducing anti-GM3(Neu5Gc) antibodies.58-61,63 A positive correlation between the development of such antibodies and patient survival was found.65 Despite the lack of direct cytotoxicity by P3 mAb,66 the antibodies generated by the anti-idiotypic vaccine were able to directly kill GM3(Neu5Gc)-expressing cells.90 Furthermore, these antibodies induced a 14F7-like type of cell death, including the formation of the membrane lesions on target cells, even at 4°C.91

Other Examples Of Non-Proapoptotic Cytotoxic Antibodies

AD5–10 mAb92 recognizes DR5, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2.93 This antibody induced apoptosis in several tumor cell lines, but was not toxic to human normal hepatocytes. Interestingly, using a pan-caspase inhibitor, it was observed that AD5–10 also induced non-apoptotic cell death, as shown by the absence of the typical DNA fragmentation and the simultaneous demonstration
of phosphatidylserine exposure and staining with the cell membrane-impermeable dye propidium iodide.\textsuperscript{92}

A prostate stem cell antigen (PSCA)-specific mAb, named IG8, was shown to directly kill antigen-expressing cells without caspase activation or apoptotic DNA fragmentation.\textsuperscript{94} A cytotoxic activity with the same features was observed for alenizumab over chronic lymphocytic leukemia cells. Binding of this mAb caused the aggregation of glycolipid-enriched domains and the triggering of a cell death pathway.\textsuperscript{95}

Although cells treated with the AD5–10 mAb and the pancaspase inhibitor exhibited a particular morphology,\textsuperscript{96} the appearance of membrane lesions in these examples was not reported.

**Future Directions**

A critical unanswered question regarding non-proapoptotic direct cytotoxicity-inducing mAbs is why some of them only kill in vitro a small proportion of target cells, in spite of recognizing almost the whole population. Deciphering their mechanism of action at the molecular level, could help to design combined strategies to increase their cytotoxic activity, e.g., by simultaneously targeting accessory molecules participating in the cell death pathway. Currently, data on the cellular processes triggered upon antigen binding are scarce. It would be particularly interesting to describe the formation of the spectacular membrane lesions induced by these mAbs. This cytotoxic activity would contribute to other better described mechanisms such as ADCC and CDC, increasing their potential for immunotherapy of malignancies and autoimmune diseases. Also, it would be useful to determine whether cells dying this way are able to activate the adaptive immune system, i.e., whether this is an immunogenic type of cell death.

**Acknowledgments**

This work was supported by the Center of Molecular Immunology.
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