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Review

A tale of the monoclonal anti-CD20 antibodies, in tribute to prof. Waclaw Szybalski (1921–2020)*

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Technical advances that lead to the era of targeted therapeutics demanded several milestones that were reached in the second half of the previous century. Professor Wacław Szybalski was the first one to perform a stable gene transfer in eukaryotic cells. To do so, he used his own designed system consisting of HPRT-deficient cells and HAT selective medium. Moreover, the first-ever hybridoma cells were also constructed by Wacław Szybalski’s team. These spectacular achievements made him not only a forerunner of immunotherapy, as hybridoma and their selection by the HPRT-HAT system turned into a crucial technical step during production of monoclonal antibodies (mAbs). Herein, we present a story of anti-CD20 mAbs, one of the most successful lines of anticancer drugs. When looking back into history, the prototypic mAb rituximab was considered the biggest step forward in the therapy of B-cell malignancies. Nowadays, the second and third generations of anti-CD20 mAbs are approved in clinical use and numerous breakthrough studies on immune effector mechanisms were conducted with the aforementioned immunotherapeutics as a model.

Keywords: monoclonal antibodies, immunotherapy, rituximab, CD20 antigen

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*This paper is dedicated to Professor Wacław Tadeusz Szybalski on the 100th anniversary of his birth

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ALL, acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; B-CLL, B-cell chronic lymphocytic leukemia; B-NHL, B-cell non-Hodgkin lymphoma; CDC, complement-dependent cytotoxicity; CHOP, cyclophosphamide, hydroxydaunorubicin, vincristine/oncovin, and prednisone; HAMA, human anti-mouse antibodies; HAT medium, medium containing hypoxanthine, aminopterin, and thymidine; HPRT, Hypoxanthine-guanine phosphoribosyltransferase

INTRODUCTION

Antibodies are the major players within the humoral immune response. Their robustness stems from their ability to bind a specific epitope that may cause direct effects on the antigen-bearing cells, as well as from the existence of the Fc domain which triggers effector mechanisms of innate immunity, such as the complement activation, phagocytosis, or antibody-dependent cellular cytotoxicity (ADCC) (Winiarska et al., 2011a; Okrój et al., 2013a). Yet, being effective against numerous pathogens, antibodies may be used against malignant cells as long as they express tumor-specific antigens or differ from their non-transformed counterparts by abnormal expression of tumor-associated antigens (Murawski & Pfreundschuh, 2010). Proper choice of a molecular target for anticancer antibodies impacts the efficacy and safety of immunotherapy. Therefore, one must use antibodies of defined specificity to ensure on-target effects. Monoclonal antibodies fulfill this demand best, as they are produced by a hybridoma derived from a single clone of B cells, which normally undertake allelic exclusion to ensure monospecificity towards a unique epitope. Nowadays, monoclonal antibodies are commonly used for medical, diagnostic, or research purposes (Stasiłojć et al., 2016; Tobinai et al., 2017). However, obtaining them was not trivial fifty years ago, which is underlined by the fact that the Nobel prize in medicine and physiology was awarded in 1984 to Georges J. F. Köhler and César Milstein for “the principle for production of monoclonal antibodies”. The undoubted technical advance that led to the production of antibodies of predefined specificity would not have been made unless several other equally important, but less appreciated milestones had been achieved, including those performed by prof. Szybalski and his coworkers.

MILESTONES OF HYBRIDOMA TECHNOLOGY

The production of non-specific antibodies in hybridoma cells, which are fusions of myeloma cells and normal lymphocytes, was first described in 1973 (Schwaber & Cohen, 1973). An improved method for the production of specific, monoclonal antibodies was introduced two years later by Köhler and Milstein (Köhler & Milstein, 1975). Importantly, this Nobel prize-awarded success was preceded by a sequence of discoveries that brought the understanding of immune system organization, and processes leading to antibody production by B cells, as well as inventions of molecular tools and techniques. The third Nobel prize winner from 1984, prof. Niels Jerne formulated two breakthrough theories: the first one entitled “Natural-Selection Theory of Antibody Formation” published in 1955 (Jerne, 1955), explaining that an organism has an inborn capacity of creating wide diversity of antibodies, but their selection for massive production depends on actual needs, i.e. the antigen that invade and selects the antibody of the best fit. The second theory, from 1971, entitled “The somatic generation of immune recognition”, came near to the idea of processes which are nowadays known as a positive selection of thymocytes and affinity maturation (Jerne, 1971; Forsdyke, 1995). The actual hybridoma technology is based on the fusion of a B cell, providing a defined specificity of antibodies, and a tumor cell, providing an unlimited number of cell divisions in continuous in
vitro culture. This essential concept in monoclonal antibody manufacturing became reliable only after previous research on myeloma cells, a fusion-inducing agent (Sendai virus), and the construction of a system for the selection of cells that underwent hybridization. Other methods than natural generation of myeloma cells was first described by Michael Potter in 1962, who discovered that injection of adjuvants containing mineral oil and heat-inactivated bacteria into the peritoneal cavity of BALB/c mice resulted in formation of neoplastic cells (Potter & Boyce, 1962). Later, cell culture conditions for maintenance of myeloma cells were set up in 1970 (Horibata & Harris, 1970). The technique originally used to perform the fusion of hybridoma partners by using the Sendai virus was made possible by the earlier work of Yoshio Okada, who demonstrated ability to induce a somatic cell fusion by using this virus (Okada & Kadokoro, 1963). In 1962 prof. Wacław Szybalski and his wife Elizabeth demonstrated the first-ever successful and stable gene transfer in a eukaryotic cell that restored the missing metabolic enzyme (Szybalski & Szybalski, 1962; Bigda & Koszalka, 2013). Besides this achievement, Szybalski provided a tool for selection of transformed cells, i.e. the HAT-HPRT system. HPRT is an enzyme that enables purine synthesis from hypoxanthine when de novo synthesis is blocked. The HAT selective medium is composed of hypoxanthine, thymidine (an alternative source of pyrimidines), and aminopterin – a blocker of the canonical pathway of nucleotide synthesis. Only cells with functional HPRT are able to continue growth in the HAT medium (Bigda & Koszalka, 2013). The HAT-HPRT system was adopted into the process of monoclonal antibody production, where HPRT-deficient myeloma cells are used as partners for fusion with B cells, and the HAT medium plays a role as a selection factor for hybrids made of two heteroplastic cell types.

THE RISE OF IMMUNOTHERAPY FOR LYMPHOPROLIFERATIVE DISEASES

Since the concept of monoclonal antibody-based immunotherapeutics against tumor cells became popular, numerous patents and trials appeared in the 80’s of the previous century. However, only a few of them turned out to be promising, mostly due to obstacles connected to the delivery of the monoclonal antibody to the tumor, poor induction of the host cytotoxic mechanisms, evasion of the immune response by malignant cells, and toxicity/side effects (reviewed in: Grossbard et al., 1992a; Grossbard et al., 1998; Uchida et al., 2004). Based on a case report, its role in the development of an immune response to T-independent antigens was postulated (Kuijpers et al., 2010), and other studies suggested its role in calcium signaling (Uchida et al., 2004; Okroj et al., 2013a), but the results of a recent study put this theory in question and propose that it has an influence on actin polymerization, cell movement and response to homeostatic chemokines (Kozlova et al., 2020). Nevertheless, even if CD20 does not fulfill the definition of a tumor-specific antigen, it possesses a number of advantages, such as a high level of expression, relatively low internalization potential, and high persistence on cell membrane (Okroj et al., 2013a). Since being discovered in 1980, it has become probably the best so far described target for therapeutic antibodies (Stashenko et al., 1980; Marshall et al., 2017). The story of therapeutic anti-CD20 antibodies began in 1997 when the Food and Drug Administration approved the first-ever antitumor monoclonal antibody ritux-
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imab, a chimeric mouse-human specimen consisting of a human IgG1 heavy chain and a mouse variable region (Maloney et al., 1997). Rituximab was tested either in a monotherapy or in combination with established chemotherapy regimens, e.g. CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine/ Oncovin, and prednisone). After completion of a series of clinical trials in the early years of the 21st century, introduction of rituximab as a frontline drug was named as the biggest step forward in the treatment of B cell lymphomas undertaken in recent fifty years (Murawski & Pfreundschuh, 2010). Indeed, 10-year follow-up of diffuse large B-cell lymphoma patients treated with CHOP, with or without rituximab, revealed significant differences in progression-free survival (36.5% vs. 20%) and overall survival (43.5 vs. 27.6%), respectively (Coiffier et al., 2010). Still promising, but not that spectacular results, were obtained for patients with B-cell leukemias, which are characterized by lower expression of CD20 when compared to lymphoma cells, and a higher level of endogenous complement inhibitors (Okroj et al., 2013b; Marshall et al., 2017). The suboptimal killing of certain types of malignant B cells, as well as the number of patients refractory to rituximab, pushed forward research on next generations of anti-CD20 antibodies and further studies on their effector mechanisms.

NEW ANTIBODIES, NEW KNOWLEDGE

Despite possessing only two relatively small loops as the only extracellular parts, CD20 became a molecular target for dozens of different monoclonal antibodies that were developed. Intriguingly, antibodies of the same class with partially overlapping epitopes exerted different effector mechanisms. While appealing, these novel anti-CD20 specimens served as models for studying the prerequisites necessary for efficient engagement of the host’s immune responses, as well as direct effects of CD20 ligation. The cytocidal activity of anticancer antibodies may stem from activation of the host immune effector mechanisms, such as the complement system, mononuclear phagocytes or antibody-dependent cellular cytotoxicity (ADCC) mostly mediated by NK cells (Cragg & Glennie, 2004; Beers et al., 2008), as illustrated in Fig. 1. Alternatively, antibodies may exert direct effects on signaling pathways leading to induction of programmed cell death (Pedersen et al., 2002; Awasthi et al., 2015). Aggregation of B cells by antibodies may also lead to reactive oxygen species-induced cell death (Honeychurch et al., 2012).

Engagement of certain effector mechanisms by a given antibody became a basis for classification of the anti-CD20 mAbs as type I and type II specimens (Winiaraska et al., 2011b). Type I mAbs are strong activators of the complement system and poor inducers of direct cytocidal effects, in contrast to type II mAbs, which predominantly interfere with cellular signaling cascades. Properties of the antibody, such as the proximity of the binding site to the cell membrane, ability to cluster the target antigen into lipid rafts, off-rate values, and structural features determine the downstream effects (Stasiloj et al., 2016), and therefore type I/II classification originally reserved for the anti-CD20 agents can be extended to many other anticancer mAbs. Based on the abovementioned classification, rituximab is an example of type I antibody. The next generation, fully human anti-CD20 antibody, ofatumumab, which was approved for clinical use in 2009 (Marshall et al., 2017), exhibits a more pronounced manifestation of class I characteristics. Similar to rituximab, ofatu-

Figure 1. Mechanisms of action of the anti-CD20 therapeutic antibodies: ADCC, CDC, and direct cytotoxicity. The CD20 molecules are indicated as ginger spots on the surface of the target cell, antibodies are depicted as red tetrameric molecules bound to CD20, C1q is a complement component initiating the classical complement activation pathway leading to the formation of MAC complex in the target cell membrane, blue rods on the membrane of natural killer (NK) cells (example of ADCC effector cells) are receptors for Fc fragments of antibodies.
mumab contains human IgG1 Fe portion and relocates antigen-antibody complexes into the lipid rafts, but it binds an epitope localized more proximately to the cell membrane (Winiarska et al., 2011b). On the other hand, obinutuzumab, another IgG1-containing specimen, shows type II characteristics and exemplifies the third generation of anti-CD20 antibodies that involves engineering of the Fe part. Obinutuzumab was modified in a way that disables fucose attachment to side polysaccharide chains. Such afucosylated antibody ensures stronger interactions with Fe receptors, thereby enhancing ADCC and antibody-dependent phagocytosis (Freeman & Sehn, 2018). Moreover, obinutuzumab exerts killing of the target cell by inducing homotypic adhesion and triggering caspase-independent, lysosome-dependent cell death (Alduaij et al., 2011).

The increasing number of either type I or type II antibodies anticipate the question of how to select the optimal therapy for the individual patient and what are the mechanisms of resistance. Effectors of the host innate immunity are robust, but not infinite, and as such prone to exhaustion, as exemplified by either an in vitro model of lymphoma cells challenged by a repeated dose of ofatumumab (Beurskens et al., 2012), or by a clinical observation that patients’ response to rituximab was significantly improved when supplemented with fresh-frozen plasma or purified complement components (Kennedy et al., 2004; Klepfish et al., 2009; Xu et al., 2011). Another reason for resistance to type-I mAbs is endogenous inhibition of the host effector mechanism by tumor cells (Jurianz et al., 1999). Overcoming these evading strategies by excessive application of antibodies is not an optimal solution due to antibody-dependent loss of molecular target by internalization (Beers et al., 2010) or shedding (Dahal et al., 2018). Importantly, selection of the tumor cell population devoid of target antigen and then resistant to therapy can be rescued by application of two different therapeutics which recognize different targets. This attempt was successfully demonstrated for the combination of ofatumumab (anti-CD20) and alemtuzumab (anti-CD52) (Baig et al., 2012, 2014), as well as for the anti-CD5 and anti-CD20 specimens (Klintgaard et al., 2013). On the other hand, some of the therapeutics may exhibit antagonistic effects, e.g. inhibitors of SRC kinases and anti-CD20 mAbs, as shown in (Winiarska et al., 2014). Nonetheless, the existence of several therapeutic options is beneficial and offers the possibility to use them in synergistic tandem with better efficacy than each agent administrated alone. Also, type II monoclonal antibodies, such as obinutuzumab which supports a direct cytoidal effect, become more popular as frontline drugs (To binai et al., 2017). We (Felberg et al., 2020) and others (Middleton et al., 2015) demonstrated that a number of patients with B-cell malignancies prescribed with type I anti-CD20 antibodies cannot mount efficient complement-dependent cytotoxicity due to primary or secondary deficiencies, and therefore might gain more benefit from application of type II immunotherapeutics. Similarly, a personalized medical attempt should take under consideration the tumor burden and polymorphisms in Fcγ receptors as parameters critical for optimal therapeutic schedule and composition (Cartron et al., 2002; Boross et al., 2011; Beurskens et al., 2012).

CONCLUDING REMARKS

The long tale of therapeutic monoclonal antibodies began more than 50 years ago not only thanks to the work of Nobel-prize winners Köhler and Milstein, but also many other scientists, including prof. Wacław Szy balski, who contributed to making this scientific and medicinal opus real. Through the last decades, we have witnessed a boom in antibody-based anticancer drugs, with anti-CD20 as the first and probably the best-characterized immunotherapeutics. We not only have specimens capable of exerting different effector mechanisms, but we also gained knowledge on how to engineer them to promote certain types of response, which may turn pivotal in personalized medicine. Apparently, this is not the end of progress since new spectacular findings were made recently, with anti-CD20 antibodies as the model. The first Science paper from 2014 has revisited our view on type I/type II antibody’s requisites (Diebold et al., 2014). The authors demonstrated that introduction of mutations into the constant portion of the heavy chain, inducing formation of non-covalent hexamers, can enforce strong complement activation by antibodies that were completely devoid of this feature when unmodified. The second Science publication from 2020 demonstrated the structures of full-length CD20 in complex with rituximab, ofatumumab or obinutuzumab, and explained the details of the antigen-antibody binding that are critical for different downstream effects of these antibodies (Kumar et al., 2020). As this knowledge seems to be adaptable beyond the anti-CD20 antibodies, the abovementioned studies will help to rationally design new immunotherapeutics dedicated to unmet medical needs.

REFERENCES


