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Commentary

Symmetry breaking: bispecific antibodies, the beginnings, and 50 years on

Gert Riethmüller

Institute for Immunology, Ludwig-Maximilians-Universität, München D-80336, Germany

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The archetypes

The era of modern antibody therapy begins with Rodney Porter's Nobel Prize-winning report on the basic structure of the immunoglobulin molecule. Using papain for digestion of a 7s rabbit antibody preparation, he obtained two almost identical antigen-binding fragments (Fab) that blocked antigen precipitation by the parent antibody while a third, easily crystallizable fragment (Fc) was found inactive (1). From a historian's viewpoint, it is striking that, almost simultaneously with these first insights into the antibody's structure and function, the idea of an artificially constructed bispecific antibody was born. Without a clear image of the now heraldic **Y** and its underlying tetrameric symmetry, Alfred Nisonoff had the vision of combining two different antigen binding sites in one molecule. He had used pepsin instead of Porter's papain to generate univalent Fab fragments that specifically inhibited antigen precipitation. Discussing his findings, he speculated about the future experiments, "to attempt to prepare antibody of mixed specificity" (2). It took him just another year to realize the idea of a $F(ab')_2$ molecule with dual specificity: this he obtained under mild reoxidation from a mixture of univalent fragments of anti-BGG (bovine gamma globulin) and anti-OVA (ovalbumin) antibodies (3). Several publications later and in collaboration with Hugh Fudenberg, Nisonoff elegantly proved the bispecificity of the reassociated $F(ab')_2$ by direct visual evidence (4). Coupling BGG and OA to human and chicken red cells, respectively, the authors demonstrated under the microscope how the two easily distinguishable red cells got agglutinated by the bispecific fragment.

Besides Nisonoff's early bispecific fragment antibody, one may cite a much older bispecific antibody, not man-made, whose biological effects had already been described in allergic patients in the 1940s because of its peculiar blocking activity on other antibodies; its true nature, however, had remained completely unknown. Part of the mystery was solved when Rob C. Aalberse and co-workers found elevated IgG4 antibodies in sera of beekeepers who were chronically exposed to phospholipase of bee venom (PLA2). These full-size IgG4 antibodies were "functionally monovalent," i.e., they blocked other PLA2 antibodies (5). The antibodies had exchanged Fab arms and consisted of two heavy and light chain pairs, each one derived from a different IgG antibody, as was only later discovered. The stochastic nature of the posttranslational formation of bispecific IgG4 molecules could later be demonstrated *in vivo* with recombinant IgG4 antibodies against defined allergens; excess irrelevant IgG4 prevented the formation of hybrid antibodies almost completely (6). The anti-inflammatory activity of the functionally monovalent IgG4 was shown in a rhesus monkey model with experimental myasthenia gravis. Whether the recently described broad spectrum of IgG4-related diseases in man is causally related to the Fab arm exchange remains to be demonstrated (7). The mechanism of IgG4 Fab arm exchange has been extensively studied by Aalberse and colleagues with a sensitive real-time FRET assay suggesting that the exchange occurs *in vivo* under specific local redox conditions (8). This story—retold here as an aside without any conceit of hindsight—and the newly recognized systemic condition of IgG4-related disease may hold some clues for a better understanding of therapy with bispecific antibodies.

This extension of the bispecific story into much earlier eras was deeply hidden when Nisonoff and Fudenberg were proving the bispecificity of the $F(ab')_2$ by agglutination experiments. Their vision anticipated in a way the action of today's recombinant bispecific antibodies designed to retarget effector cells at cancer cells. However, the Nisonoff approach would have remained without any traceable consequences had Lloyd Old not given it a serious try. Together with Ulrich Hämmerling, Old used the original $F(ab')_2$ procedure to develop a bispecific antibody addressing mouse immunoglobulin and ferritin, thus generating a universal reagent to detect immunoglobulin on the surface of mouse lymphocytes by electron microscopy (9). The low yield of the original Nisonoff-Rivers method apparently prevented its broader application.

1985-1995: The bispecific explosion

About 20 years later—during which time the hybridoma technique of Georges Köhler and César Milstein had come into widespread use—Henry Paulus and co-workers, using monoclonal antibodies, improved the yield of bispecific $F(ab')_2$ through a chemical coupling procedure (10). A similar coupling of F(ab') fragments based on tandem thioether molecules was introduced by Martin Glennie and co-workers shortly thereafter (11).

Milstein himself had entered the bispecific arena two years before Paulus with the hybrid hybridoma approach, later called quadroma, an allusion to the four genomes in the final hyperploid cell (12). Because of the motley assortment of various H and L chains in the quadroma supernatant, the yield of the one desired bispecific pair of H/L chains was extremely low. Following the Lloyd Old trail, Milstein and Cuello applied the isolated anti-somatostatin x anti-peroxidase bispecific antibody for a one-step electron microscopic detection of somatostatin in brain and pituitary. The influence of that report can hardly be underestimated: it set off a string of papers on various bispecific monoclonals.

In 1984, little more than one year after Milstein's paper, Michael Bevan and co-workers submitted their decisive work on a bispecific antibody that aimed at recruiting T cells for cell-directed cytotoxicity (13). For addressing T cells, they used a monoclonal antibody against the T cell receptor, and for tumor targeting, an antibody against a Thy-1 alloantigen on a leukemic cell line was employed. The two antibodies were coupled by SDS, a heterobifunctional cross-linker. The impact of this paper on the whole field was mainly due to the enormous redirected cytotoxicity that was unleashed by the bispecific antibody. The report impressed a group of investigators that had been working for some years on targeted cellular cytotoxicity. They had employed heteroconjugated antibodies to engage Fc receptor-bearing cells for antibody-dependent cell-mediated cytotoxicity (ADCC) against defined target cells.

Thus it is no wonder that in less than four months after the appearance of the Staerz/Bevan report, David Segal, one of the protagonists of the "ADCC community," and his group published their version of a T cell-recruiting bispecific antibody. In 1984, just one year before Staerz and Bevan, they had already employed the SPDP-based coupling procedure to generate $F(ab')_2$ heteroconjugated fragments focused on Fc receptor-bearing cells (14). With this experience, it was a matter of a few months to adapt the whole procedure to construct a bispecific $F(ab')_2$ consisting of an anti-human CD3 arm, derived from OKT3, and an anti-murine H-2^k-alloantigen arm. Human anti-HLA cytotoxic T cell clones were used as effectors against murine K^k-positive tumor cells. The new bispecific $F(ab')_2$ antibody, though equipped only with univalent binding arms, exhibited a similar degree of cytotoxicity as the hybrid full-sized antibody of Staerz and Bevan (15). The lysis of the xenogenic targets by the human T cell clones was compelling evidence that MHC compatibility was completely dispensable.

In the wake of these two 1985 reports, a flurry of papers appeared all trying to apply the new powerful tools to engage all sorts of effectors against various target cells. In a follow-up to their original report, Staerz and Bevan showed that bispecific antibodies could inhibit growing tumors *in vivo* and that virus-infected cells were excellent targets for this approach (16, 17).

1989-1997: Five international conferences on bispecific antibodies and targeted cellular cytotoxicity

Within the short period of four years after 1985, the bispecific movement had gained so many followers that the leaders of the ADCC field, Michael W. Fanger and David M. Segal, could convene a "First International Conference on Targeted Cellular Cytotoxicity and Bispecific Antibodies" that assembled about 120 aficionados in the autumn of 1989 in Annapolis, Maryland. That indeed two scientific worlds had then come together is revealed by the report that appeared after the meeting; its title read, "Going both ways: bispecific antibodies and targeted cellular cytotoxicity" (18). During the fast succession of the four conferences at Seillac in France (1990); Rosa Marina in Puglia, Italy (1992); Key West in Florida (1995); and Volendam, The Netherlands (1997), it was the term "bispecific" that gained general acceptance. What had started as a small local conference with fewer than 150 scientists culminated as "The 5th World Conference on Bispecific Antibodies" in 1997 at Volendam near Amsterdam that attracted a big crowd of participants. Though a large part of the program was devoted to reports on clinical trials with bispecific antibodies, protein engineering based on recombinant DNA techniques received center stage attention and opened the meeting.

The explosive growth of the bispecific field that had occurred in the short decade before the Volendam meeting is vividly reflected in a comprehensive review by Christoph Renner and Michael Pfreundschuh entitled: *Tumor Therapy by Recruitment with Bispecific Antibodies* (19). Of the 150 or so listed references, about 40 were original reports on bispecific antibodies. The authors, both clinicians themselves, had performed interesting preclinical and clinical trials in Hodgkin disease administering an anti-CD16 x anti-CD30 bispecific antibody prepared by the hybrid hybridoma method. The story of these investigators is quite revealing with respect to the difficulties of that period. In two small therapeutic trials, each on about 15 end-stage Hodgkin patients, the authors had obtained evidence for some clinical activity; in the second one of these trials, they made the interesting observation that all three patients with objective responses had received the antibody as a 4-day continuous infusion (20, 21). Unfortunately, the trial had to be closed down prematurely because the commercial partner stopped the production of the bispecific antibody due to the extremely low yield of antibody from quadroma supernatants. While the Hodgkin disease trial was closed because of the lack of antibody, the Genentech group of Paul Carter had already published the CH3 heterodimerization technique based on the Knob-in-Hole principle (22). A similar incongruity between bench and bedside existed in the field of bispecific fragment antibodies. Understandably, the clinic was lagging behind, while at the bench, antibody generation had already progressed to produce single-chain Fv molecules, single-chain bispecific antibodies [e.g., diabodies by Holliger *et al.* (23)], and single-chain tandem Fv bispecifics (24, 25).

After the 5th World Conference on Bispecific Antibodies: the rapid rise of therapeutic antibodies

Despite the various advances in antibody engineering reported at this splendid "5th World Conference on Bispecific Antibodies," a kind of disillusionment was spreading after the meeting, mainly because of the poor clinical results, but also because of the observed toxicities, and last but not least due to the change of interest within the industry supporting the expensive clinical trials. Follow-up meetings, such as the one in 1999 at Southampton organized by Martin Glennie, had given up the term *bispecific* in the title and were concentrating mostly on therapeutic full-size antibodies. Compared with the advances in antibody engineering and the relatively straightforward translation into the clinic, the bispecific antibody approach, relying mostly on the recruitment of T lymphocytes or of Fc receptor-bearing cells, looked so much more risky. Indeed, progress in engineering antibodies had moved very fast. It took just two years from first chimerization to humanization. Nevertheless, as noted by Alain Beck, it took twelve years from the invention of the phage display in 1990 to the first fully human antibody to arrive in the clinic routine (26). Further steps in the intact antibody field were the development of better antibody-drug conjugates and the refinement of variable domains with affinity-matured complementarity-determining regions (CDRs) and fine-tuned Fc fragments.

Though these antibody engineering advances also spilled over to the bispecific field, new clinical trials were initiated almost exclusively with intact antibodies because of their easier production mode and less complicated and risky translation

into non-cancer indications such as immunological or infectious diseases. The massive introduction of engineered therapeutic antibodies, antibody-drug conjugates, and monospecific antibody fragments into clinical practice, during this short decade around the turn of the century, will undoubtedly figure as one of the great success stories of modern medicine (27). Toward the end of 2010, Janice M. Reichert, an impartial chronicler of the fast evolving antibody field, estimated that about 30 antibodies and antibody fragments had been approved for various indications, ranging from infection and autoimmunity to inflammation and cancer (28).

A plethora of bispecific formats and one single bispecific antibody approved

Despite the overwhelming success of therapeutic antibodies, research and development in the bispecific field did not come to a standstill. In contrast, with the new recombinant DNA tools for protein engineering at hand, a growing number of laboratories started to work on bispecific antibodies, and within a few years, they turned out new formats galore. An impressive account of this development is given in a recent book edited by Roland E. Kontermann (29), who gives an encyclopedic introduction into the bispecific field with a list of more than 150 references. In view of the abundance of different formats reaching from miniaturized versions such as domain antibodies and nanobodies up to somewhat bizarre decavalent and tri- and tetraspecific antibodies, it is somewhat ironic that the only bispecific antibody that so far has gotten approved for clinical use is a rather low-tech antibody derived from rat/mouse quadroma. The antibody owes its existence to the serendipitous observation by a young investigator who tried to isolate a hybrid bispecific antibody from such quadroma (30). Due to the different affinities of the H chains of the two species for protein A and a preferred intraspecies L/H pairing, Horst Lindhofer managed to prepare a highly enriched rat/mouse bispecific antibody of the desired configuration. Obstinately and almost single-handedly, he established a GMP-proof production facility and, with the help of a big corporation, initiated a clinical development program of the anti-EpCAM x anti-CD3 bispecific antibody (catumaxomab) that led to approval for the restricted indication of malignant ascites. The antigen of catumaxomab, now called EpCAM, has had a checkered past as a tumor-associated antigen since it was found to be ubiquitously expressed on all simple epithelia (31).

Like EpCAM, most of the antigens used for tumor therapy with bispecific antibodies were differentiation antigens that rarely showed a tumor-related increased or altered expression such as CD19, CD33, CEA, MCSP, EphA2, or EGF receptor. One of the few exceptions is HER2/neu, that—when amplified in the genome—plays a "driver" role in breast cancer progression. Thus far, however, none of the several anti-HER2/neu bispecific formats has attained approval. The recognition of differentiation antigens present on cancer stem cells has opened a new approach for several organ cancers such as prostate, breast, and pancreas cancers. Interestingly, EpCAM is now being found on the majority of epithelial stem cells or tumor-initiating cells. Besides the group of oncofetal antigens, there is the large group of Cancer/Testis antigens exhibiting an interesting tumor-associated expression, but unfortunately they are almost exclusively expressed in the nucleus or cytoplasm. What makes a membrane antigen a good antigen for attack by retargeted T cells? The answer is by no means clear. As recently shown by Claudia Bluemel and co-workers for a bispecific antibody against melanoma chondroitin sulfate proteoglycan (MCSP), the epitope distance to the target membrane had a major influence on the induced lysis (32).

The CD19 x CD3 single-chain bispecific antibody: learning lessons on T cell engagement the hard way

When the single-chain bispecific format of Mack *et al.* (24)—later also employed for the design of CD19 x CD3 antibody (blinatumomab)—was presented for the first time at the aforementioned Volendam conference in 1997, it was met with considerable skepticism, if not with open disbelief (33). How could it be that a univalent binding to CD3ε by a bispecific antibody sets the whole signal-transducing machinery in motion and initiates the cytolytic process?

As to the activation of naïve or resting T lymphocytes by antigen, one adamant dogma had been established during the decade from 1985 to 1995, namely that T cells require a second signal besides the TCR/antigen-mediated signal for stimulation. This essential costimulatory signal had to be delivered through membrane receptors like CD28, CD40, and others. Therefore, in the wake of the Staerz/Bevan and Perez/Segal reports on T cell recruitment, it was tacitly accepted that secondary signals were involved and were absolutely required to engage and activate T cells by bispecific antibodies. According to the opinion leaders of the day, costimulator ligands on tumor cells like B7 were needed to trigger resting T cells to become cytotoxic (34). Indeed, Gundram Jung and collaborators could show that the cytotoxic effect of heteroconjugates consisting of OKT3 and a full-length melanoma antibody was greatly enhanced with a second heteroconjugate containing an anti-CD28 antibody arm (35).

However, with the particular anti-CD3 as part of the CD19 x CD3 bispecific, Dreier *et al.* showed in a lymphoma xenograft model that human T cells did not express any detectable activation markers and yet became highly cytotoxic for the target *in vivo* (36). In the meantime, it had become clear that bispecific antibodies, while establishing contacts between effectors and targets, are aggregating their engaged antigens on the two opposite cell membranes into a kind of microcluster or patch whereby the two CD3ε heterodimers come in close contact and, by induced conformational change, start the downstream signaling process through the transmembrane bundle of the TCR complex. Whether the CD3ε pairs are dislodged from the TCR via the univalent binding CD3 bispecific antibody, as was suggested by previous work with crystallized OKT3/CD3ε, has not yet been analyzed with bispecific antibodies (37).

Work by Patrick Baeuerle and co-workers has shown bispecific antibodies apparently can build *bona fide* immune synapses between T cell and target cell without TCRs establishing a close fit with a congruent peptide-MHC complex. They also demonstrated that additional multipoint attachments effectuated by accessory molecules like CD8 or CD4 were not required for lysis to occur (38). The dispensable role of the αβTCR in T cell stimulation is undoubtedly a big advantage for the bispecific approach since MHC molecules are frequently lost or downregulated on cancer cells. Acknowledging this remarkable activity of the bispecific single-chain antibody—in essence a tandem array of two Fv domains—the acronym "BiTE" for *Bispecific T cell Engager* was appropriately chosen by Baeuerle.

In view of the remarkable potency of the BiTE format, the question arose of how it might compare with the anti-tumor activity of full-length therapeutic antibodies. A particular interest for such a comparison was focused on cetuximab and panitumumab, a prominent pair of antibodies that were directed at the EGF receptor and were approved for the treatment of colorectal cancer (CRC). It had recently been discovered that these antibodies exerted their therapeutic efficacy mainly through a blockade of the EGF receptor. Furthermore, a retrospective analysis had shown that CRC patients with mutated *KRas* and *BRAF* genes did not benefit from antibody treatment.

In order to compare the BiTE format as closely as possible with a full-length antibody, the group of Baeuerle and Kufer cloned the variable Fv domains of cetuximab and panitumumab and inserted them into the BiTE format. In side-by-side *in vitro* and *in vivo* experiments on *KRas*- and *BRAF*-mutated CRC cell lines, the authors showed that the lytic T cell attack triggered by the two bispecific antibodies at subpicomolar concentrations was completely independent of the intracellular mutations. Cetuximab and panitumumab, however, were inefficient when faced with tumor cells having a deficient RAS-RAF-MAPK signaling axis (39). These experiments underscore the role of T lymphocytes as main defenders against intracellular foreign invaders; they, and not antibodies, are the ones entrusted with the task to eliminate the organism's own cells when they have become breeding places for viruses. The self-killing is a fine-tuned process that at the end initiates the cascade leading to programmed cell death of the transiently contacted target (40).

The new pharmacology of CD8 effector T cells in cancer therapy

Envisioning the CD8 T effectors as the essential cytotoxic partners for the anti-CD3-containing bispecific antibodies, one has to note that these migrating T cells do not exhibit chemotactic behavior toward uninflamed tumor cells, therefore the meeting of the threesome of T lymphocyte, target, and bridging antibody comes as a stochastic event. The probability of the trio to meet is largely dependent upon the local frequencies of the effectors and targets, as well as on the overall concentration of the antibody. Given this scenario, it is evident that multiple local constraints differing from organ to organ and from compartment to compartment will greatly impact the conditions so that all three players come together at the right place and right time. This will have quite some bearing on the treatment of solid tumors with bispecific antibodies.

After years of trial and error, continuous infusion had been found to be the most efficient regimen, meeting at best the constraints of the scenario designed above. In view of the very short half-life in blood, the small antibody (55 kD) had to be given ample time to penetrate into the interstitial space and maintain adequate concentration there. In a trial on non-Hodgkin lymphoma patients, these deliberations were confirmed by the observed dose-effect kinetics. For example, at 5 μg/day/patient, the lowest tested dose of blinatumomab in adults, B lymphocytes or B lymphoma cells were depleted only in bone marrow. Lymphoma cells in other organs or in enlarged lymph nodes required much higher doses in order to show a response (41). Systemic cytokine release well known from other bivalent anti-CD3 antibodies does not occur at concentrations of the CD19 x CD3 (blinatumomab) in the low picomolar range; local release of cytokines is restricted to sites where contact between effector T cells and target occurs.

It is of interest to note that among the many and various cancer trials with different bispecific antibodies, two successful trials stand out because of their indication areas: these were the peritoneal cavity with ascites of different epithelial cancers in the case of catumaxomab, and the bone marrow with residual acute lymphocytic leukemia (ALL) in the case of blinatumomab. In both situations, a barrier to free access existed neither for T lymphocytes nor for the bispecific antibodies. In contrast, bone marrow had previously been identified as a "nest for migratory memory T cells" by Di Rosa and Pabst (42). In hindsight, it comes almost as no surprise that 80% of ALL patients with minimal residual disease cleared their bone marrow of the residual leukemic blasts after long-term continuous infusion of 15 μg antibody/day. These few remaining ALL cells can be detected with a sensitive and highly specific reverse transcription polymerase chain reaction (RT-PCR) test. This test allows the detection of a very low copy number of RNA molecules specific for the individual leukemic cells of a given patient. As to the prognostic power of this test, 82% of ALL patients with a positive RT-PCR test relapse within a rather short period. All of the treated patients had failed various treatments, particularly 12 of 16 responding patients (43).

In summary, the two most successful trials with bispecific antibodies were the ones devoted to the "easiest" indications. The conclusions for the design of future bispecific strategies may be drawn accordingly. With regard to the question of the best regimen of administering bispecific antibodies, many factors influencing CD8 T cell stimulation are unknown. Does long-term continuous infusion with a BiTE antibody resemble chronic stimulation as it occurs in chronic virus infection? The remarkable rise of CD8 T cell effector memory cells in peripheral blood occurring under continuous infusion of BiTEs speaks in favor of such an interpretation. During viral infection, T cells undergo proliferative expansion and may contract only later into a pool of memory cells (44). In certain virus infections, such as cytomegalovirus (CMV), the amplification of the CD8 T cell subset has been inflationary. If that would occur after continuous stimulation with a bispecific antibody, does it entail increased anti-tumor efficacy in later stages of the treatment? Another outcome of chronic virus infection is loss of T cell function, such as loss of autocrine cytokine secretion, that leads to a state of T cell exhaustion characterized by PD-1 or CTLA-4 expression. Is this condition reversible as suggested by Barber and colleagues (45)? Another unsolved question concerns the role of CD4 T cells and their influence on the CD8 T effector dynamics when patients are treated with several cycles of T cell-recruiting bispecific antibodies. In another chronic virus infection model, CD4 cells have been found to rescue exhausted CD8 T cells (46).

The future: competitors, combinations, and challenges

Presently, more than 40 different formats of bispecific antibodies have been designed and produced in the laboratory and more are undoubtedly to come. How many of these constructs and which ones will be administered to patients is hard to foresee. Will the DART (Dual-Affinity Re-Targeting) format—a variation of the diabody format—be more successful than BiTEs? For indications other than cancer cell elimination, bispecific nanobodies or domain antibodies seem to have a brighter future. The hybrid hybridoma approach has lately seen substantial improvements with respect to easier recombinant production and higher yields. Recently, a novel heterodimeric Fc

platform has been proposed that supports the design of full-length bispecific antibodies via alternating segments of IgG and IgA within the CH3 domains (47). Others have improved on the Knob-in-Hole technique and developed a common light chain approach adapted to the CDR on each of the two different H chains (48). These full-length antibodies also seem to trigger an active immunization process against associated tumor antigens via the Fc fragment's affinity for antigen presenting cells such as dendritic cells (49). Induction of an adaptive humoral immune response was also observed after treatment with the described catumaxomab (50). Whether this active immunity has a clinically relevant effect on tumor growth or tumor cell persistence remains to be studied.

Bispecific antibodies will undoubtedly take further advantage of the progress made with immunomodulating antibodies. It is well established that the majority of T effector memory cells is preferentially localized in nonlymphoid tissue (51). Immunomodulating antibodies like anti-CD40 or anti-CTLA-4 impact on the migration and distribution of these extralymphoid migratory T cells. First combinations of such immunomodulators with anti-tumor antibodies have been reported. With regard to solid tumors—still the greatest challenge for intact and bispecific antibodies—it was recently shown that under therapeutic CTLA-4 blockade tumor-infiltrating CD8 T cells can increase up to 100-fold also in those patients who did not respond to the antibody treatment with shrinkage or rejection of their tumor (52). Therefore it is foreseeable that a fine-tuned control of CD8 T cells may become an effective adjunct to future cancer therapy with bispecific antibodies.

Abbreviations

BiTE, Bispecific T cell Engager

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Contact

Address correspondence to:

Gert Riethmüller, M.D.
Institute for Immunology
Ludwig-Maximilians-Universität
München D-80336
Germany
E-mail: gert.riethmueller@med.uni-muenchen.de