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Can antibodies be "vegan"? A guide through the maze of today's antibody generation methods

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ABSTRACT

There is no doubt that today's life sciences would look very different without the availability of millions of research antibody products. Nevertheless, the use of antibody reagents that are poorly characterized has led to the publication of false or misleading results. The use of laboratory animals to produce research antibodies has also been criticized. Surprisingly, both problems can be addressed with the same technology. This review charts today's maze of different antibody formats and the various methods for antibody production and their interconnections, ultimately concluding that sequence-defined recombinant antibodies offer a clear path to both improved quality of experimental data and reduced use of animals.

Introduction

The development of methods for the generation of antibodies has forever changed research, diagnostics, and therapy. Behring's antitoxins (now called polyclonal antibodies),[1] Milstein and Köhler's hybridoma technology to generate monoclonal antibodies,[2] and finally the development of antibody phage display by Breitling, McCafferty, Barbas and colleagues[3–5] deeply influenced medicine, immunology, molecular biology, biochemistry, and biotechnology. Immunofluorescence literally changed the way we look at biological molecules[6] by allowing a single protein to be identified through a microscope. Building on developments that started in the mid-1980s, antibodies are now used in more than 160 new medications (www.antibodysociety.org/resources/approved-antibodies/) to treat cancer, infections, autoimmune and other diseases, with many more expected.[7] The reason for this widespread use is the unique ability of antibodies to recognize a specific molecule among tens of thousands of other biomolecules and bind to it with extraordinary affinity. This particular capability has contributed to the evolutionary success of longer-living animals including ourselves, as it provides a very effective weapon system against parasites, microbes and viruses that otherwise would rapidly overwhelm us. As these small organisms evolve much more rapidly than we do, our bodies routinely generate antibodies recognizing molecules that the human species may never have encountered before. This marvellous capability makes antibodies useful for research, as we can generate immunoglobulins to almost all molecules of suitable size that provide a defined structure and a minimum of interactions to assure high-affinity binding. This is illustrated by the creation of antibodies to molecules not generally considered antigens, such as Buckminsterfullerene[8] or polyethylene glycol.[9] This vast structural variability of millions of different molecular interaction surfaces originates from a clever loop arrangement at the tips of the immunoglobulin's arms.[10] The structural diversity is achieved by DNA fragment recombination and even new DNA synthesis in B cell development.[11] Later, during antigen-dependent B cell differentiation and clonal selection, every individual antibody-producing cell undergoes clonal expansion, and can further improve the antibody it produces by somatic hypermutation.[12]

Today, we can imitate all essential steps of this antibody generation process *in vitro*,[14] allowing some restrictions of our own immune system to be bypassed, such as the bias introduced by the necessity to present peptides on the major histocompatibility complex (MHC), or the limited options for heavy-chain/light-chain pairing in individual B cells. This approach also allows antibody generation to be done without any animal experiments,[15] which has led companies to market them as "vegan antibodies". Qualifying antibodies as vegan would mandate that generation, analysis, and production are achieved without the use of animals or animal-derived materials. There are currently quite a number of different ways to discover new antibodies against a desired target and produce them in sufficient quantities (Figure 1). Many cross-connections between these methods further complicate the picture, so that the use of animals or animal-derived materials is not always readily apparent. Even de novo generated antibody sequences designed by artificial intelligence (AI)[16] rely on the structural guidance of nature's antibody repertoire, as the AI systems cannot be successful without training on the

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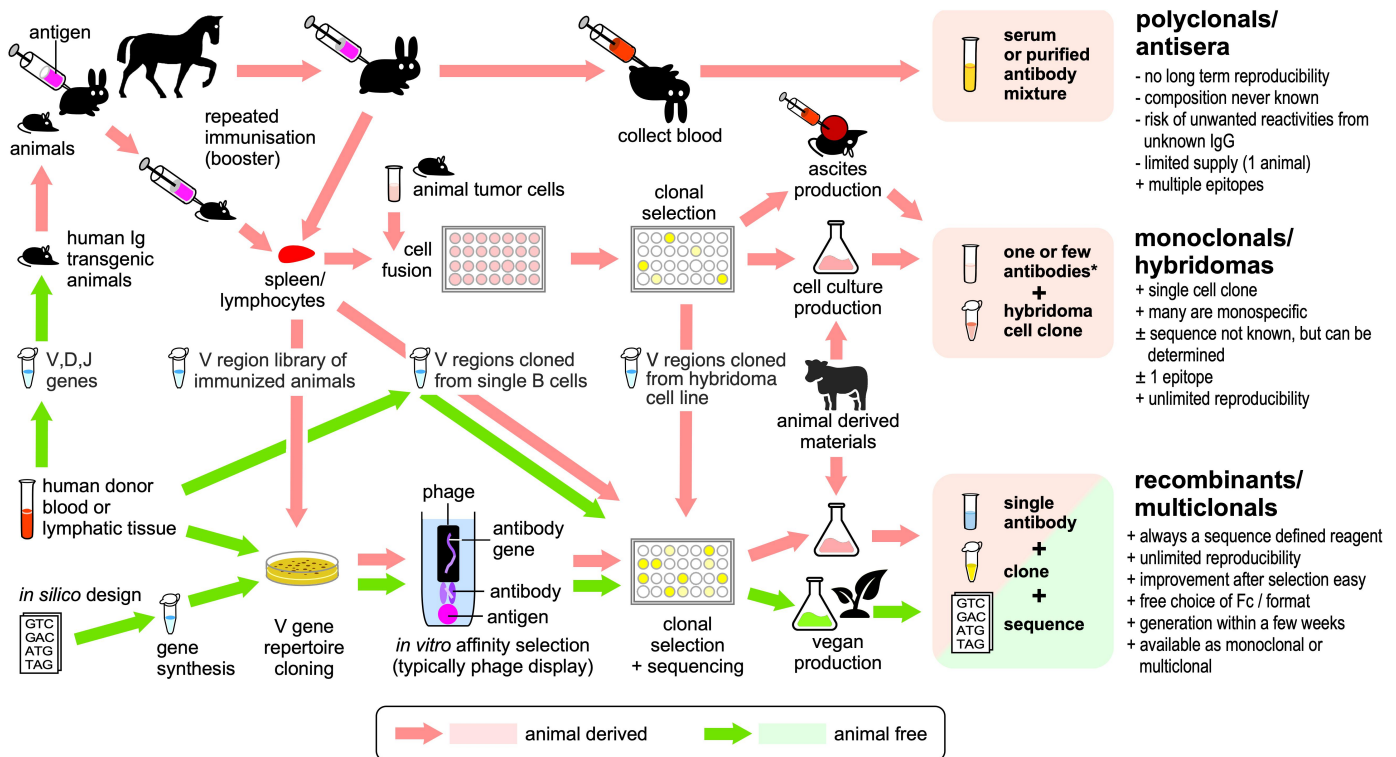
Figure 1. The intricate paths of antibody generation. Organisms are shaded in black. Importantly, recombinant antibodies are not always non-animal derived, as today the various advantages of the recombinant format are utilized both by animal- and non-animal-derived antibodies. Vast antibody variable region gene libraries, which can be obtained from human donor blood or by in silico design, offer animal-free access to antibody genes. However, the use of gene libraries does not always guarantee animal-free products, as they can be used in transgenic animals or obtained after animal immunization. It is also often overlooked that, after the initial animal-free generation of an antibody clone, animal materials may be used to produce these antibodies in useful quantities. Conversely, today any recombinant animal- or non-animal-derived antibody could also be produced in a vegan cell culture system. Since this map shows several paths consisting entirely of green arrows, "vegan" antibodies are indeed a reality. *, see[13]

sequences and structures of countless animal-derived immunoglobulins. However, this strict approach does not reveal actual animal use in a specific antibody generation project. In the analysis and discussion below, materials such as donor blood-derived human antibody DNA or the human HEK293 production cell line are not considered animal-derived because they do not originate from animals, which typically include mice, rats, hamsters, rabbits, horses, goats, sheep and guinea pigs but may also include monkeys, chickens, dogs, llamas, and sharks.

How to make antibodies

For the first 85 years of their use, antibodies were only available in the form of blood serum or polyclonal immunoglobulin fractions obtained from it. Although these crude antibody preparations are always undefined in respect of their true specificity spectrum, they saved the lives of children with diphtheria and other diseases,[17] and substantially expanded our understanding of histology and cell biology.[6]

The Use of polyclonal antibodies, however, has drawbacks, as only 0.5–5% of the immunoglobulins in a polyclonal reagent bind to their intended target.[18] Polyclonal serum is composed of an antibody mixture resulting from all immune responses of the donor animal, which may be independent from the immunization with the desired antigen. Further, this composition varies from batch to batch, because the immune response is

different in every individual animal[19], and even between subsequent blood samples taken at different times from the same animal.[20] The additional specificities are of course unwanted, but never mapped, resulting in poorly defined reagents. While affinity chromatography on the antigen to enrich the desired specificities is possible,[21] this is only offered for a small fraction of the catalog products. Using the flow-through of an affinity chromatography on antigens that must not be recognized by the final polyclonal product allows the reduction of cross-reactivities. This approach is most prominently used to remove inter-species cross-reactivity of secondary antibodies.

Despite these approaches to improve their performance, the shortcomings of polyclonal antibodies are responsible for numerous false scientific conclusions.[18,22] Fifteen papers published on the dementia-related protein C9ORF72 that generated 3,500 first-layer citations and 66,000 secondary citations involve work with an antibody that does not recognize C9ORF72.[23] Many antibodies underperforming in a validation study of more than 600 antibodies were used in a large number of published articles.[24] Poorly characterized and ill-defined antibodies were in large part responsible for the failure to replicate the scientific results of 47 of 53 landmark preclinical studies.[25] In a western blot analysis of 13,000 antibodies, only about 45% recognized their expected targets.[26] While this could be explained in part by the denaturation of epitopes, Berglund et al. found that more than half of around 6,000 routinely used commercial monoclonal and polyclonal

antibodies recognized more than their specified targets.[27] Numerous authors have lamented the substantial amounts of money wasted per annum for antibodies that do not work or produce false results and the much higher follow-up cost for our society.[28] Human lives are also at stake: In addition to research work, clinical trials and diagnostic tests for cancer were based on the use of antibodies that have since proven to not bind to the assumed target or were nonspecific.[29–33] Knowing all of this, it is surprising that polyclonals still represent the largest product group for research antibodies (Figure 2). But why are research products that obviously are *"littering the field with false findings"*[34] still so popular? Sadly, this is not driven by their performance, but mainly due to their much lower price and lack of knowledge about the alternatives.[15]

The polyclonal nature of serum-derived antibodies also has a positive aspect: immune responses of the immunized animals typically lead to the expansion and differentiation of several antigen-specific B cells that in most cases cover different epitopes on the antigen of interest. The resulting serum therefore offers better chances to recognize the antigen in different experimental settings where parts of the epitopes may have changed due to denaturation (i.e., in western blots or fixed tissue sections), and the recognition of several epitopes on the same molecule can increase the detection signal in an assay.[35] These advantages can be exploited, as the presence of the unknown fraction of unwanted IgGs in polyclonal preparations is excluded. Multiclonal antibodies,[36] composed of an exactly defined mixture of sequence-defined recombinant antibody clones, provide the polyclonal advantage, resulting in elimination of false positive signals, and they provide lower background because they do not contain any potentially misleading off-target binders.[37]

The development of hybridoma technologies in 1975 was a substantial advance in antibody generation methods.[38] Hybridoma generation relies on the fusion of two cells: a B cell encoding the desired antibody and a tumor cell that provides the immortality necessary for unlimited future cultivation to produce the monoclonal. Monoclonal antibodies originating from a single immortalized B cell revolutionized many areas of life sciences. For example, the knowledge explosion in molecular immunology was initiated and driven by a rapidly growing collection of monoclonal antibodies to lymphocyte surface markers. Monoclonal antibody clones literally defined cluster of differentiation (CD) antigens, allowing researchers for the first time to distinguish between the many different cell types of the adaptive immune system, which until then had all looked the same under the microscope.[39] They also enabled the development of the first therapeutic monoclonal antibodies from the 1980s onwards.[40]

For decades, monoclonals provided the gold standard for specificity, as they represented a huge improvement in specificity over polyclonal serum products. Recent results, however, explain why some monoclonals are not monospecific.[27] First, two or more $V_H DJ_H$ or $V_L J_L$ recombination patterns of immunoglobulin heavy or light chains have been found in many single B cells.[41] Second, due to the cell fusion that leads to hybridomas, these are initially tetraploid and carry four different antibody alleles. Hybridoma cells grow like cancers and are not regulated by a surrounding immune system. As a result, the number of chromosomes found in hybridomas varies widely; some hybridomas were shown to have developed up to 50 additional chromosomes.[42–45] A multicentric study that sequenced expressed antibody genes of 185 hybridomas, including a large number of viable research catalog products, showed that only 68.1% of the analyzed hybridomas expressed only one of their four alleles, while the rest produced additional antibody chains (mainly light chains), which resulted in the secretion of more than one antibody by many truly monoclonal cell lines.[13] This led to false positive reactivities and lower sensitivity.

This problem can be solved by cloning the variable regions of the antibodies from the hybridomas to generate recombinant versions. Many methods are available to do this, most prominently by PCR[46,47] or next-generation sequencing.[48,49] Once the correct pair of V_H and V_L is identified, these can be re-inserted into the genes encoding the chains for a full IgG and efficiently produced in mammalian cell culture.[50] Nowadays, both transient production methods with HEK293 cells[51,52] and stable cell lines, typically CHO,[53] are well established for recombinant IgG and often provide yields of over 5 g/l. All recombinant immunoglobulins resulting from such a clone have the same structure and specificity. Importantly, this recombinant approach can also be used if the hybridoma is contaminated with bacteria or fungi, produces antibodies with undesirable glycosylations, does not adapt to the cultivation medium or is no longer sufficiently productive. These

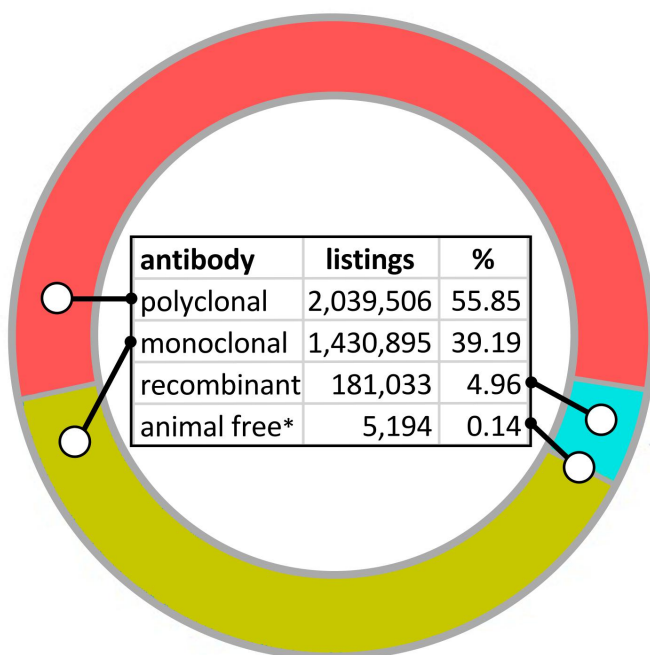| antibody	listings	%
polyclonal	2,039,506	55.85
monoclonal	1,430,895	39.19
recombinant	181,033	4.96
animal free*	5,194	0.14

Figure 2. Biocompare.com search for different research antibody products, as of February 2024. The search engine could not distinguish between different products derived from the same original antibody preparation, e.g., with different labels or packaging sizes. As different labels are more commonly offered for secondary antibodies, which are mostly polyclonals, this segment may be slightly overrepresented. * Only 27 antibodies made by animal-free methods were listed in this database; this label was obviously not yet provided for all recombinant antibodies made by these methods. The recombinant antibody website https://afa.ehstaging.net/listed 5194 different animal-free antibody products by the same date, which would still represent only 0.14%.

problems[54,55] were usually solved by a passage of the hybridoma line through the abdomen of a living mouse,[56] where they generate an ascites tumor that is very painful for the laboratory animals.[57]

Due to the knowledge of their sequence, recombinant antibodies are therefore always structurally defined reagents, in contrast to the vast majority of monoclonal and polyclonal research antibodies available from commercial suppliers. As a result, recombinant antibodies improve the reproducibility of research because they can be provided as long as the sequence information is known – a remarkable logistic advantage compared to the efforts necessary to keep hybridomas available in liquid nitrogen tanks. A substantial fraction of experiments published with monoclonals cannot be repeated because the hybridoma clones are no longer available. In contrast, databases collecting antibody sequences have been established to support unlimited reproducibility of experiments with recombinant antibodies.[58]

Beyond IgG: the choice of different antibody formats

Once available, antibody variable region DNA can be cloned into vectors encoding various other formats or other Ig types. Most methods using *E.coli* as expression host and most *in vitro* selection methods use just antigen-binding portions of the antibodies, like single-chain variable fragments (scFvs),[59,60] antigen-binding fragments (Fabs)[61] or scFab.[62] Regardless of the format in which the antibodies were originally selected, the final application format is usually different today, as format conversions are uncomplicated in recombinant production systems. Various antibody formats are currently used for research (Figure 3).

IgG is the most commonly used antibody format in research. IgG molecules offer two antigen-binding sites and thus improved apparent affinity in all assays where antigen density and localization support avidity effects. Their Fc region is easily recognized by secondary antibodies for detection, and protein A enables efficient purification of all molecules containing an Fc moiety in one step.[63] Polyclonal antibodies are often supplied as IgG-enriched fractions, and most hybridoma-derived antibodies are supplied as purified IgG fractions. However, some hybridomas only produce IgM,[64] which provides higher avidity due to their 10-12 antigen-binding sites, but must be carefully checked for cross-reactivity, as IgM represent an earlier stage of antibody maturation in the B cell and typically have lower monovalent affinities per antigen-binding site.

Antibody fragments can be either generated on the protein level by chemical/proteolytic cleavage of animal-derived IgG preparations, or by respective design of the antibody genes for recombinant production.[65] F(ab')2 are generated by enzymatic cleavage of immunoglobulins from animals or humans.[66] They have two antigen-binding sites of an IgG but can be used in assays that would be interfered with by the presence of Fc parts, e.g., when using material with a high content of Fc receptors. Due to their smaller size, they penetrate tissue more efficiently. They allow double staining when combined with an IgG, as they can be detected by anti-Fab-specific secondary antibodies that do not interfere with anti-Fc-mediated antibody detection.

Fragments antigen-binding, F(ab), also known as Fab, can be produced either by chemical reduction of the disulfide bonds in the hinge of F(ab')2,[67] or recombinantly in *E. coli* or eukaryotes.[68] Fabs provide monovalent antigen binding, which in some assays can result in much weaker signals compared to the IgG from which they are derived due to the lack of avidity. Recombinant Fab are expressed from two separate open reading frames (encoding the light chain and the corresponding Fd fragment). They are commonly used in phage display and mammalian cell display, and are the most efficient format for the expression of functional antibodies by yeast surface display.[69] Single chain fragments antigen binding (scFab) are a variant of Fabs in which the light chain and the corresponding Fd fragment (VH and CH1) are joined by a peptide linker to be expressed from a single open reading frame.[62] They can only be produced recombinantly. Their main advantage is that only one gene fragment needs to be transferred when subcloning the Fab fragment into other
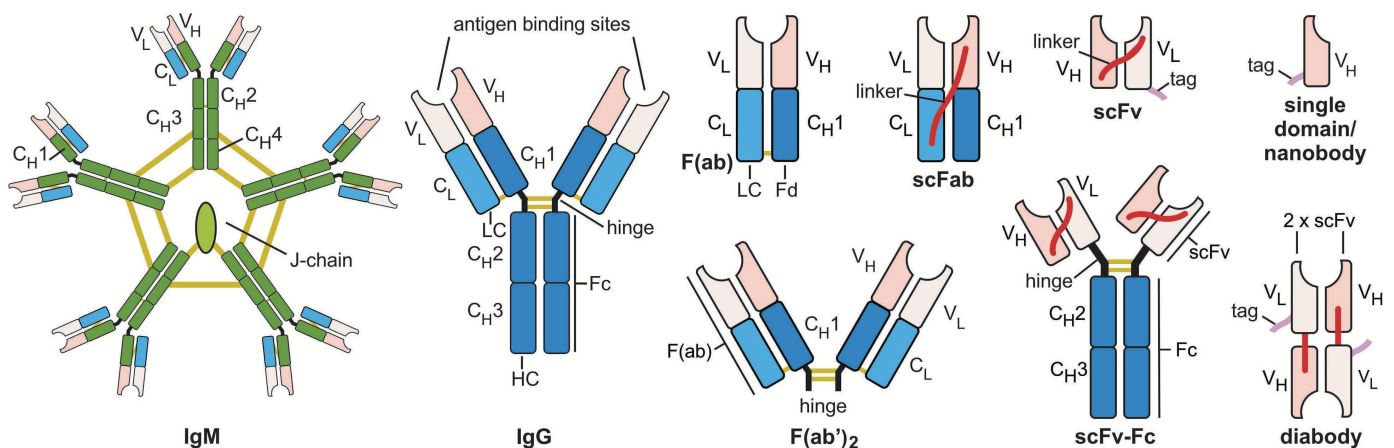

Figure 3. Antibody formats most frequently used in research assays: IgM, Immunoglobulin M; IgG, Immunoglobulin G; F(ab')2, divalent fragment antigen binding; F(ab), fragment antigen binding, now usually named Fab; scFab, single chain fragment antigen binding; scFv-Fc, single chain fragment variable::Fc fusion protein; scFv, single chain fragment variable; diabody, dimerized scFv; and single domain antibodies/nanobody. Constant regions are colored dark blue (γ chains), green (μ chains), or light blue (κ or λ chains); variable (antigen binding) regions in pink. Yellow lines indicate disulfide bonds, red lines peptide linker, purple lines peptide tags. C, constant region, V, variable region, HC, heavychain; LC light chain. Elements are not drawn to scale.

formats.[70] However, scFab:Fc fusions (single chain immuno-globulin G, scIgG) tend to form higher-order aggregates.[71] ScFab have also been used to generate mammalian cell surface expression libraries for *in vitro* selection using transposon technology.[72]

Single chain fragment variable:Fc fusion proteins (scFv-Fc) offer the advantage of full compatibility with common second-ary anti-Fc fragment antibodies for their detection and generic protein A affinity chromatography for their purification, while requiring cloning of only one gene fragment encoding both the VH and VL regions that form the complete antigen-binding site,[73] for example, after *in vitro* selection from an scFv gene library. ScFv-Fc makes up a large part of the non-animal derived catalog antibodies, as their subcloning and production are less costly compared to conversion to full IgG, while they can be used just like IgG.

Single chain fragment variable (scFv) is the smallest stable constructs to contain the full antigen-binding site.[59,60] As they are produced with high yield in *E. coli*, they are often used in antibody phage display libraries. ScFv provides monovalent antigen binding, but because they lack structural stabilization by the constant regions, some of them can form bivalent homodimers (diabodies), which may have a higher apparent affinity due to avidity. This can lead to a different performance of the antibodies in assays where avidity plays a role compared to assays that only detect monovalent binding. Diabody for-mation can be forced by shortening the linker between the V regions.[74] This provides a simple way to produce bivalent antibodies in *E. coli*. However, it should be noted that in diabodies the flexibility in the spatial orientation of the two antigen-binding sites (paratopes) is severely restricted due to their rather rigid conformation, whereas the antigen-binding sites of naïve IgG have much greater freedom of movement to contact their antigens due to the flexibility of the hinge region.[75] As a result, the gain in apparent affinity by avidity may differ between a diabody and an IgG with identical paratopes.

Single domain antibodies[76] and nanobodies[77] consist of only half of the antigen-binding site of an IgG (which comes from the heavy chain), as they have no light chain. They can be produced efficiently in *E. coli*. Some variants of them can efficiently fold in the cytoplasm,[78] making them in particular suited to be used as intrabodies.[79] Nanobodies derived from heavy-chain only IgG antibodies found in the *Camelidae* family have an enlarged complementary-determining region 1 (CDR1) and an elongated CDR3 loop that facilitates the binding of antigens with a groove and allow binding to a different structural spectrum of antigens than IgG.[80]

ScFvs, diabodies and nanobodies can be used for research purposes, but usually require an additional incubation step for detection, as they do not have constant regions/Fc fragments that can be recognized by conventional secondary antibodies. Although the conserved framework sequences within V regions of some scFvs and nanobodies may be recognized by some preparations of anti-IgG polyclonals, this is not guar-anteed for every single fragment due to their high variability. Therefore, peptide tags (usuallybetween 4-15 amino acids resi-dues in length) are added to the scFv or nanobodies to enable a generic detection and/or purification procedure. The c-myc

tag is often used for this purpose. It can be detected either with the animal-derived monoclonal antibody Myc1-9E10[81] or the affinity/stability-enhanced "vegan" Hypermyc antibody.[82] His-tags provide simple generic purification,[83,84] the FLAG-tag provides both purification and detection,[85] Strep-tag[86] or Avi-tag[87] allow to use avidin and streptavidin-based reagents, and many other tags are available. To avoid the use of tags, scFv can be converted into Fab or IgG by subcloning. However, this can affect stability and antibody function,[88] and some scFvs loose binding affinity when converted to full IgG format. Therefore, *in vitro* selections using surface display systems should be performed in formats as close as possible to the final application.[89]

To be useful in the laboratory, antibodies must not only bind to an antigen with high specificity and affinity but also have additional properties such as long-term stability, low aggregation in solution, high expression yield for low-cost production and more. These "developability" factors[90] must be taken into account when producing antibodies for the low-margin research markets. While the "natural" IgG format is considered the most inherently stable variant, not all *in vitro* selection systems are capable of producing complete IgG mole-cules, which usually require mammalian cells for efficient production and correct folding. Gene libraries with a semi-synthetic design, in which natural complementarity-determining regions (CDRs) of antibodies are grafted into antibody frameworks of known "well-functioning" antibodies,[91] or fully synthetic designs based on very stable scaffolds[92] offer a way to generate good research reagents with minimal effort.

Since some scFvs and the widely used scFv-Fc fusions derived from *in vitro* selectionshave a higher tendency to aggregate, this parameter must be checked early inthe antibody selection process to exclude molecules with developability liabilities. It is important tonote that these are properties of individual scFv molecules, as the scFv formatis by no means always less stable. Very robust scFv reagents can be obtained directly from libraries[93] or by *in vitro* optimization.[94] This is also illustrated by many clinically used fusion proteins that contain scFvs, like the chimeric antigen receptors (CAR) where the largest group of extracellular-binding regions are scFvs,[95] or bispecific antibodies[96] and immunotoxins.[97] For research applications, however, conversion to a full IgG is often the chosen solution to provide full compatibility to the assays established for animal-derived antibodies. This conver-sion can also be used to add functionalities that are not easily available from animal-derived antibodies. For example, by cloning the variable regions of different antibodies into vectors encoding Fc parts of different species, antibodies originating from the same library can be converted to appear as IgG from different species, and detected by differently labeled detection reagents. Combined in one experiment, their use in combina-tion with Fc-specific anti-rabbit, anti-rat, anti-human, or anti-mouse secondary antibodies for detection allows for multi-color analyzes not possible in the same way with a set of mouse monoclonals or rabbit polyclonals.[98] Conversion to other formats like IgA, IgE or IgM are also possible in case the respective functionality is needed. Even non-canonical amino acids can be inserted at defined positions during cell

culture production to provide functionalities not achievable with animal-produced antibodies.[99,100] Last but not least, the availability of antibody variable (V) region DNA has allowed construction of a vast and growing zoo of non-IgG-like fusion proteins. The resulting bispecific antibodies,[101] immunotoxins,[102] or CAR-T cells[103] have already provided many new and successful therapeutic approaches.

Making antibodies without using animals

All the advantages of recombinant immunoglobulins also apply to antibodies developed entirely *in vitro* because these methods are based on antibody genes that are recombinantly expressed in cultivated cells, so that their genetic blueprint is always available. The first successful and still prevalent method to generate antibodies completely without immunization and independent from a living immune system is antibody phage display.[3–5] It employs highly diverse gene libraries of rearranged immunoglobulin genes (10^{10}-10^{11} different clones), obtained either from B cells (often human), chemical synthesis or by a mix of both. These libraries encode a collection of variable (V) regions of antibodies that structurally determine the paratope (antigen-binding surface) of the antibody, and thus its specificity and affinity. "Universal" (also named "naïve") antibody gene libraries can be made without the use of animals.[104] These libraries are cloned into a phagemid vector[4] which, when present together with a helper phage genome in the same *E. coli* cell, leads to the production of antibody phage that have a physical linkage of the antibody protein to the DNA encoding it. Mixtures of antibody-phage, containing billions of different functional antibody fragments, each linked to their encoding genes, are then incubated with immobilized antigen. While the vast majority of antibody-carrying phage does not interact with the antigen, a few antibody-phage are bound to the antigen and can be eluted, after washing away the unbound ones. As they are phage, after elution they are able to very efficiently re-infect *E. coli*. The antibody-phage also carry a resistance marker that allows only the antibody-gene carrying bacteria to multiply. DNA encoding antigen-specific antibody genes can easily be isolated from the resulting *E. coli* colonies. This process, called "panning", relies on individual single molecule interactions to identify the sequences of recombinant monoclonal antibodies.

The antibody DNA can be ligated into many different vectors providing a final antibody format of choice. Typically, it is cloned into suitable IgG expression vectors to produce the full monospecific immunoglobulin.[105] These immunoglobulins carry all functions of IgG from animal sources, and consequently are applicable to any assay that has been developed for animal-derived antibodies,[15] as they are structurally identical to them. More recently developed *in vitro* antibody selection methods, like yeast display[106] or mammalian cell display,[107,108] are based on the same principle: clonal selection from vast DNA repertoires using the antigen-binding function of antibodies that are physically attached to the cell carrying the encoding gene. These methods offer the advantage of glycosylation and the display of larger antibody formats including full IgG, which is extremely inefficiently expressed in *E. coli*. Although the diversity of the repertoire

of antibody genes that can be utilized with eukaryotic cell display methods still is several orders of magnitude lower than the diversity of 10^{10}-10^{11} different antibody genes typically used in phage display,[109] complex selections can be made in cell display systems that would be much more difficult to perform with phage display. For example, with appropriate labeling in the selection step of flow cytometry, both antibody expression and antigen binding can be quantified simultaneously for each individual antibody-expressing cell. With the right gating in flow cytometry, significantly higher enrichment factors can be achieved than with phage panning, reducing the number of selection rounds required. A further advantage of these approaches is that they enable integrated selection for antibody developability. Here, the mammalian display can select not only for antigen binding but also simultaneously for well-expressing and stable antibody candidates. This is best achieved by displaying the "final" full IgG format. In addition, both display methods can be combined, using a phage display library with a diversity that cannot be achieved with cellular display in the first round of selection to obtain more initial binders, followed by batch subcloning into a cellular display system for refinement [110].

Phage display and the other surface display methods allow the generation of specific immunoglobulins against everything that can bind to antibodies with reasonable affinity, as they are not restricted by the bottleneck of peptide presentation on MHC (Major Histocompatibility Complex). This allows to obtain antibodies against antigens that cannot be handled by a living immune system, like toxins or non-peptidic materials. In addition, most *in vitro* display libraries use random pairing of light and heavy chains, which means that the chance of achieving an optimal V_H/V_L match is much greater than in any B cell. This is a significant advantage compared to antibody selection in animals, where B cells can only produce two rearranged versions of the light chain per heavy chain.[111] This limitation is one reason why repeated immunizations (boosts) are necessary in animals to increase affinities by somatic hypermutation. In contrast, by using *in vitro* display libraries with random V_H/V_L pairing, similar affinity increases (>1000 ×) can be achieved without introducing point mutations, just by the identification of a better fitting light chain from repertoires of > 10^6 V_L region genes.[112] This process, named "chain shuffling", exchanges the light chain of a given antibody with the full light-chain gene repertoire before performing a new phage, yeast or mammalian display selection.[113] Since both chains of the resulting antibodies have sequences with a much lower number of mutations compared to the human germline, the probability of developing adverse immune reactions in patients treated with these antibodies should be lower than with somatically hypermutated IgG. Combined with careful adaptation of the selection conditions, even the kinetic properties of antigen binding can deliberately be changed while keeping specificity and affinity identical.[114] Of course, somatic hypermutation can also be reproduced *in vitro* by adding point mutations prior to *in vitro* selection.[94]

The complete control over the biochemical milieu at the very moment of antibody-phage selection is another advantage of *in vitro* selections.[114,115] Antibodies with pre-adapted properties can be obtained in this way. For example, the buffer

conditions of the final assay the antibody has to work in can be used during its selection, yielding only antibodies that survive these buffer conditions. Even the kinetic properties of an antibody can be adjusted: simple prolongation of the washing steps during the panning selects for antibodies with increased stability and slower dissociation.[94] Unwanted cross-reactivities can be counter-selected against by adding soluble competitors during the panning step. The other way round, sequential affinity selections on different homologous proteins can identify a common structural motive.[116] This can be used to select for antibodies that cross-react with homolog proteins from different species – a feature that is important for preclinical development that relies on animal models to demonstrate efficacy and test for target-related toxicity.

In the future, combining sequencing of a person's entire B-cell receptor immune repertoire (BCR sequencing) with deep-learning algorithms to identify disease related antibody sequences[117] or de-novo prediction of antigen binding site (paratope) structures[16] may offer additional ways to identify specific antibodies.

Why do different origins of antibodies matter?

The properties of polyclonal, monoclonal, and recombinant antibodies, in particular their specificity, false positive reactivities, recognized epitopes and reproducibility, differ significantly (Figure 1 right). More rigorous validation certainly would be beneficial for all types of research antibodies. A systematic study analyzing 614 commercially available antibodies targeting 65 protein targets[24] underlined the paramount importance of validation of every individual antibody for each application. Polyclonal, hybridoma-derived, and recombinant antibodies were validated for their performance in western blot, immunofluorescence, and immunoprecipitation. Within this set of antibodies, recombinant antibodies performed better when directly compared to hybridoma-derived monoclonals and polyclonals directed at the same protein. Also, some of the assumed advantages of polyclonals were not evident, as these did not outperform the monoclonal antibodies in immunoprecipitation and were universally worse across the three applications tested. In another study, recombinant IgG cloned from hybridoma lines performed better than the IgG purified directly from supernatants from that same monoclonal hybridoma line.[13] In conclusion, we should at least minimize the risks of false positive reactivities that originate in the undefined nature of polyclonals[118] by using monoclonal, or even better, recombinant antibodies whenever possible. Here, we should be aware that additional reactivities can be present in some hybridoma-derived antibodies despite the origin from a single clone.[13] However, the latter risk is far lower than that originating from the use of polyclonal antibodies. Luckily, the availability of suitable recombinant technologies enables the conversion of both reagent types to completely defined reagents with known polypeptide sequence.

As long as the animal that produced a polyclonal serum is still available, single B cell cloning[119] or V region libraries generated from lymphatic tissue or blood combined with phage or yeast display[120] can be used to save the information

on the immune response against the antigen used for immunization, by generating one or several individual recombinant antibody clones, which should provide similar specificities completely without the contamination by a large fraction of unknown IgG. Most importantly, this conversion allows unlimited production of these antibodies in the future, in contrast to the limited amount of serum-based polyclonals, which is defined by the blood volume that a single animal can provide over its lifespan. Even the multi-epitope recognition advantage of polyclonal antibodies can be restored in recombinant form by using multiclonals[37] that avoid the disadvantages of serum products.

The advantages of the recombinant formats and vast gene libraries inspired the creation of many approaches that use a mix of animal-based and *in vitro* methods. As a consequence, antibody phage display is often not animal free. Antibody gene repertoires isolated from immunized animals are frequently used to combine the advantages of the *in vitro* selection process with the results of immunizing a natural immune system. For example, nanobodies can be identified from phage display libraries generated from the blood cells of immunized llamas or camels.[121]

Another approach is offered by transgenic animals, which were originally created to avoid adverse side effects induced by a patient's immune response against murine therapeutic antibodies.[122] While genetic engineering allows for many different approaches to convert a mouse monoclonal to a more "human-like" sequence (humanization),[123,124] a more direct approach was made possible by cloning the entire antibody gene repertoire of humans into mice, rats or rabbits which had their own IgG genes knocked out.[125–127] Immunization of these "human IgG-transgenic" animals leads to the production of antibodies using the human sequence repertoire – a major source of the currently used therapeutic antibodies.

By amplification of the antibody genes from single B cells,[119] recombinant versions of antibodies can be generated very quickly, as recently demonstrated during the SARS-CoV-2 pandemic.[128] The race to produce therapeutic antibodies against the coronavirus also highlighted the potential of the phage display antibody to generate antibodies against antigens previously unknown to the human immune system. Before the first COVID patient materials were available in Europe, universal phage display libraries provided SARS-CoV-2 neutralizing antibodies within a few weeks, as the only material that was needed was the sequence information of the antigen.[129] So, antibody phage display demonstrated the capability to identify binders against newly emerging pathogens more rapidly than any animal immunization-based approach can,[130] and today's naïve/universal antibody libraries for *in vitro* surface display now match immune sources for the affinities of antibodies that can be obtained.[91,131–133]

With all of these advantages, one might wonder why recombinant antibodies are not used much more frequently. In fact, while they represent less than 5% of the research reagents (Figure 2), all monoclonal antibody products currently used in human therapy are recombinant. The explanation for this dramatic difference in the acceptance of an obviously superior technology is complicated but is clearly based on their intended use. A report by the European Union Reference

Laboratory for Alternatives to Animal Testing (EURL ECVAM)[15] found that a combination of a lack of knowledge about recombinant formats and scientific misconceptions, including preconceptions about new methods stemming from issues encountered during the early days of the technology, contributed to the causes. The most important factors, however, are the limited availability and the usually higher price. Even antibodies derived from hybridomas, which have been available for almost half a century and have much better properties, have not been able to replace polyclonals as research reagents for the same reason (Figure 2), as they are around 5–10 times more expensive than polyclonals. Generation of new recombinant antibodies currently costs at least the same or even more than hybridoma generation, mainly due to their still small market share. Wider adoption of recombinant research reagents that meet the optimal standards available today would therefore require significant investments to broaden the industrial base for their supply, which should also lead to a significant reduction in price. Notably, the current research catalog prices of animal-free recombinants are not higher when compared to animal-derived antibodies.

Vegan antibodies?

The technology currently available can provide DNA blueprints for highly specific antibodies with high affinity without the need for animal experiments. In particular, antibody phage display has yielded many research antibodies.[134,135] Non-animal-derived antibodies are used in diagnostics without being identified as such, and a growing number of antibodies obtained by animal-free methods are used as therapeutics.[136] Consequently, the ECVAM report concluded that methods that do not involve animals can now yield research antibodies as good as animal-derived antibodies,[15] and thousands are already available in catalogs today (Figure 2; see also https://afa.ehstaging.net/).

Nevertheless, it is not always easy for researchers who want to avoid animal use to understand which antibodies fulfill this criterion. Importantly, *in vitro* antibody production methods are not a priori animal-free. To identify those approaches that would qualify as "vegan" according to the practical criteria for animal use set forth in the introduction, we first have to look at the origins of the V region DNA. Antibody V genes can be collected from the genomes or transcriptomes of non-immunized or immunized animal or human donors, or they can be synthesized chemically, typically in the form of "synthetic" libraries.[137–139] The latter are based on one or a few antibody V region genes (frameworks) taken from databases. Only the loops directly contacting the antigen (CDRs) are randomized during DNA synthesis to provide the structural diversity within the antigen binding site.[140] Even *in vitro* cell-based methods to replicate both primary and secondary antibody diversification mechanisms were conceived.[141]

While human antibody libraries usually qualify as non-animal derived, not all antibodies labeled as "human antibodies" do. They could have been produced in human IgG transgenic animals after immunization,[125–127] or originate from humanizing a hybridoma.[123,124] A pragmatic approach

in respect of minimizing animal use would be to check whether the antibody diversity originates from animal immunization or directly from an animal used for this purpose, or whether it just uses antibody structures originating from databases or from human blood donations.

Another route to recombinant antibodies uses V genes identified by sequencing hybridomas. This is a valuable path to further utilize the huge trove of historically created monoclonal antibodies. It would be detrimental to the progress of science to forego their use, since the animal experiments for their generation are finished, and if done right, no new animals are needed for their ongoing production. Instead of using the ascites method[56] to save deteriorating or contaminated hybridoma lines, recombinant production could be used. Moreover, an *"Antibody Heritage Program"*[142] to generate recombinant versions of all available hybridomas would not cause more animal use, but assure the long-term survival of valuable monoclonals and upgrade them with the advantages of the recombinant format. This would even allow their fully vegan production – of course, not qualifying them as vegan antibodies, but it would be a step to reduce animal suffering.

Although they are still rarely found in research reagent catalogs, a large number of alternative molecular scaffolds has been developed over the past decades that could replace the antibody molecule in many usual assays.[143] However, many of them need different detection systems, for example by using anti-tag antibodies, which takes away some of their original advantages and still limits their routine use in research today. All of these are initially identified by *in vitro* selection methods analogous to those used for antibody generation, and that do not require animals.

While antibody discovery from gene libraries can be done with or without animal experiments, the same is also true for their recombinant production (Figure 1). Here, sequences obtained by gene synthesis from databases or from clones established decades ago that encode the constant regions of different animals are always required to reconstruct the V regions into full IgG. Only this format is fully interchangeable for animal-derived antibodies in typical research applications.[15] While human Fc parts that meet the "non-animal-derived" criterion could be used in many applications, they are less suitable when analyzing human samples and tissues that naturally contain immunoglobulins. Consequently, recombinant IgG versions with the constant parts from animals such as mice, rats, rabbits will be needed for research. Of course, new animal experiments will never be necessary to use this constant region DNA, as all the necessary gene fragments have long been made available in commercially sourced vectors.

The actual use of animal materials in the protein production step of non-animal-derived *in vitro* generated antibodies is often much more difficult to identify. While the inhumane ascites method to produce monoclonals from hybridoma tumors in mice[56] has been used to generate monoclonals, it is not used for the production of recombinant antibodies. Nevertheless, *in vitro* production in cell culture often relied on animal materials, such as fetal calf serum in the growth medium. However, defined media that completely avoid the use of animal-derived reagents are available for both

hybridomas[144] and recombinant antibody-expressing cells.[51,52] In addition, various systems for the plant-based production of antibodies have been developed,[145] offering an undisputable vegan solution. Along the same lines, antibodies can be made in yeasts, which have proven to be particularly useful to provide defined glycosylation of the antibodies. For example, glycoengineered *Komagataella* (formerly named *Pichia) pastoris* strains were used for the production of humanized mAbs with glycosylation patterns optimized for therapeutic use.[146–148]

Various materials derived from animals may also be used in the assays that are an integral part of the discovery and production processes. Bovine serum albumin or skimmed milk powder is typically used as blocking reagents in the ELISA screening required to identify the correct clones or test the correct function of production batches. These animal-derived reagents can be replaced by chemically synthesized or plant-derived materials (Abcalis, personal communication). While we cannot completely avoid animal use in the development of therapeutic antibodies,[149] we now have a choice to go the "vegan" way of antibody making for many research applications.

The author would like to reiterate his opinion that legal restrictions on the use of animals for the production of antibodies would considerably hinder research and the development of drugs.[149] Rather, efforts should be made to improve general access to non-animal-derived antibodies, allowing more researchers to recognize the intrinsic advantages of *in vitro*-derived antibodies. Wider use of animal-free methods for the generation of antibodies should be encouraged, as the resulting use of defined recombinant reagents would be beneficial not only for the animals but also for the quality and reproducibility of our experimental results.

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