

REVIEW

From rabbit antibody repertoires to rabbit monoclonal antibodies

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In this review, we explain why and how rabbit monoclonal antibodies have become outstanding reagents for laboratory research and increasingly for diagnostic and therapeutic applications. Starting with the unique ontogeny of rabbit B cells that affords highly distinctive antibody repertoires rich in *in vivo* pruned binders of high diversity, affinity and specificity, we describe the generation of rabbit monoclonal antibodies by hybridoma technology, phage display and alternative methods, along with an account of successful humanization strategies.

Experimental & Molecular Medicine (2017) 49, e305; doi:10.1038/emm.2017.23; published online 24 March 2017

INTRODUCTION

European rabbits (*Oryctolagus cuniculus*; Figure 1) have played an important role as animal models in immunology for many decades.^{1,2} Today, rabbits are still a major source for a wide variety of monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) with broad utility. pAbs can be described as a set of different antibodies generated in response to a specific pathogen or antigen, generally targeting different epitopes. mAbs, on the other hand, contain a defined antigen-binding site (paratope) that typically binds with high affinity and specificity to only one epitope. From a pharmaceutical point of view, mAbs provide a molecularly defined and reproducible product, whereas pAbs are traditionally an imprecise mixture of different antibodies.³ As is the case for mouse and human mAbs, IgG is the most common isotype of rabbit mAbs (Figure 2).

Rabbit pAbs have been used extensively as analytical tools in biomedical research and especially for immunological techniques, such as immunohistochemistry (IHC), western blotting and flow cytometry. Rabbit pAbs have also been utilized as an important tool for food safety assessments.⁴ In addition, rabbit pAbs have been used in a clinical context. A prominent example is anti-thymocyte globulin (ATG). ATG is a mixture of purified polyclonal rabbit, horse or goat IgGs against human T cells that has been used as an immunosuppressive drug for decades. In organ and allogeneic bone marrow transplantation, ATG application causes rapid depletion of T cells, leading to a decreased risk for rejection and acute graft-versus-host disease. However, ATG does not induce long-term tolerance.⁵

Rabbit ATG is one of the most commonly used ATGs, due to its higher lymphocytotoxicity compared to horse ATG.⁶ Rabbit ATG known as Thymoglobulin (Sanofi Genzyme, Inc., Cambridge, MA, USA) was approved by the US Food and Drug Administration (FDA) in 1998. More recently, an improved rabbit pAbs cocktail targeting human leukocytes was reported as a potential immunosuppressive drug in xenogeneic (for example, pig to human) organ transplantation.⁷ In addition, one rabbit pAb is currently approved by the FDA as an *in vitro* diagnostic tool (c-Kit pharmDx; Agilent Technologies, Inc., Santa Clara, CA, USA) for the IHC-based detection of CD117 (c-kit) expression in gastrointestinal stromal tumors to aid treatment decisions.⁸

mAbs and mAb-derived antibody therapeutics^{9,10} are currently widely used to treat human diseases, such as cancer and autoimmune diseases.^{11,12} Although no therapeutic rabbit mAbs have been approved by the FDA thus far, 11 rabbit mAbs are FDA-approved *in vitro* diagnostic tools in the clinic.^{13,14} Ten of these mAbs are being used to detect the expression of tumor-associated antigens, including HER2, estrogen receptors, progesterone receptors and PD-L1. One mAb is used to detect helicobacter pylori infections. A rabbit mAb to human androgen receptor splice variant 7 has emerged as a promising tool for the detection of circulating tumor cells by immunofluorescence and IHC in prostate cancer.^{15,16} In addition, several rabbit mAb-derived therapeutics are currently being investigated in clinical trials registered at ClinicalTrials.gov. In oncology, examples include sevacizumab (Simcere Pharmaceutical Group, Inc., Nanjing, China), a humanized



Figure 1 *Oryctolagus cuniculus*. Shown is an individual with b9 κ -light-chain allotype from a pedigreed non-inbred colony of rabbits developed and characterized at the US National Institute of Allergy and Infectious Diseases, National Institutes of Health. The rabbits are currently housed at a rabbitry in Stanwood, WA, USA, owned by R & R Research, Inc. and available for custom immunization and harvests of peripheral blood, spleen and bone marrow. (Photo courtesy of Dr Rose G. Mage).

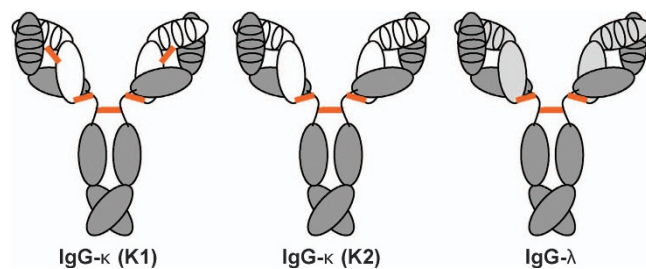


Figure 2 Schematic drawing of natural rabbit antibodies in IgG format. The ~150-kDa rabbit IgG molecule contains two identical κ (white) or λ (light gray) light chains paired with two identical heavy chains (dark gray). The light chain consists of an N-terminal variable domain (VL), shown with its three CDRs, followed by one constant domain (CL). The heavy chain consists of an N-terminal variable domain (VH), also shown with its three CDRs, followed by three constant domains (CH1, CH2 and CH3). CH1 and CH2 are linked through a flexible hinge region that has the amino-acid sequence APSTCSKPTCP (or APSTCSKPMCP in an allotypic variant) and anchors three disulfide bridges (orange) of the IgG molecule, one for each of the two light- and heavy-chain pairs, and one for the heavy-chain pair. Notably, rabbits have two κ light chains, K1 and K2. The more frequent κ light chain, K1, contains an additional disulfide bridge that links VL and CL. Rabbits of the commonly used New Zealand White strain have ~90% IgG- κ (K1), ~10% IgG- κ (K2) and <1% IgG- λ antibodies. CDR, complementarity-determining region.

rabbit anti-human vascular endothelial growth factor mAb (NCT02453464),^{17,18} APX005M (Apexigen, Inc., San Carlos, CA, USA), a humanized rabbit anti-human CD40 mAb (NCT02482168), and YYB101, a humanized rabbit anti-human HGF mAb (YooYoung Pharmaceutical Co., Inc., Seoul, Korea; NCT02499224).^{19,20} Further, chimeric antigen receptor T cells based on a rabbit anti-human ROR1 mAb²¹ have commenced clinical trials recently (NCT02706392).^{22,23} In ophthalmology, humanized rabbit anti-vascular endothelial growth factor mAb brolicizumab (Alcon, Inc., Hünenberg, Switzerland), a mAb in scFv format administered intravitreally, is being investigated in advanced clinical trials (NCT02307682 and NCT02434328).²⁴ Following these examples, a number of rabbit mAb-derived therapeutics are expected to transition from preclinical to clinical studies in the near future. Currently, only a handful of companies develop rabbit or rabbit-derived mAbs for laboratory research and for diagnostic and therapeutic applications. Some of the companies in the global market are Abcam, Inc. (Cambridge, UK; RabMab platform; through acquisition of Epitomics, Inc., Burlingame, CA, USA, in 2012); Alcon, Inc. (through acquisition of ESBatech, Inc., Schlieren, Switzerland in 2009); Apexigen, Inc. (San Carlos, CA, USA; spun out by Epitomics, Inc. in 2010); Cell Signaling Technology, Inc. (Danvers, MA, USA; currently listing >4000 different rabbit mAbs); Agilent Technologies, Inc.; MAB Discovery, Inc., Neuried, Germany; Lab Vision Corporation, Inc., Fremont, CA, USA; Thermo Fisher Scientific, Inc. (Carlsbad, CA, USA; Invitrogen ABfinity recombinant rabbit antibodies); and Ventana Medical Systems, Inc. (Tucson, AZ, USA).

What is the attraction of rabbit antibodies for the applications discussed above? Rabbits have been used to investigate immunological questions and to develop immunological techniques for >100 years. Thus, many standard procedures are established, published and validated, such as immunization and purification methods yielding high amounts of rabbit antibodies.²⁵ In addition to these practical considerations, rabbits are characterized by a variety of natural features that make their antibody repertoire very attractive for the discussed applications. First, rabbits belong to the order Lagomorpha, which is evolutionary distinct from the order Rodentia, to which, for example, mice and rats belong.^{26–29} Rabbit antibodies are able to recognize epitopes on human antigens that are not immunogenic in rodents,³⁰ increasing the total number of targetable epitopes and facilitating the generation of antibodies that cross-react with mouse orthologs of human antigens.^{31–33} This is an important aspect for basic research and preclinical investigations with, for example, human tumor xenografts, where the presence or absence of on-target-off-tumor toxicities of therapeutic antibodies provides important information prior to clinical translation. In general, rabbit anti-mouse reactivity is valuable in mouse models of human disease and has also been exploited in basic research, for example, on mouse stem cell antigens.^{34–36} Second, it has been observed that rabbits elicit strong immune responses against small molecules and haptens, which is uncommon in rodents.^{18,37–41} A third important aspect is the scarcity of inbred rabbit strains, while most mouse strains are inbred.^{1,42} It is thought that inbred strains in general elicit less diverse immune responses, which makes it more difficult to

raise strong and diverse binders.¹⁸ Correspondingly, in many IHC studies that compared rabbit and mouse mAbs to the same human antigens, rabbit mAbs consistently revealed higher sensitivity.^{43–62} In a recent study,⁶³ 1410 rabbit mAbs raised against 15-amino-acid peptides representing 100 different antigens revealed a typical affinity range of 20–200 pM (median 66 pM), as determined by high-throughput surface plasmon resonance. A fraction of rabbit mAbs even revealed higher affinities near the detection limit of 1 pM. However, the affinity range of 46 mouse mAbs (30–300 pM; median 72 pM) analyzed in parallel was not significantly different.⁶³ More direct comparisons of rabbit and mouse mAbs to a wider variety of antigens are warranted to determine a significant difference in affinity. Fourth, most strategies to generate mAbs are based on the recovery of B cells from spleen, bone marrow or blood, which are present in higher quantities in rabbits than in mice due to their overall larger body size. (The average body weight of a 3-month-old laboratory rabbit is 2.5 kg compared to 25 g for a 6-week-old laboratory mouse.) For example, 50 times more spleen B cells can be recovered from rabbits compared to mice.¹⁸ In addition, the larger blood volume of rabbits compared to mice facilitates mass spectrometry analyses of bulk serum IgG.⁶⁴ Fifth, as discussed in the next section, rabbits use different mechanisms to genetically generate and diversify their primary and secondary antibody repertoires compared to humans and mice, effectively creating a complementary set of binders for the discussed applications. The recent sequencing and annotation of the rabbit genome (OryCun2.0 assembly) has provided new insights into this unique antibody repertoire.^{1,65,66}

ONTOGENY OF RABBIT B-CELL AND ANTIBODY REPERTOIRES

The development of the rabbit B-cell repertoire significantly differs from that of other mammals. The current model proposes a three-step process (Figure 3) consisting of the neonatal B-cell repertoire generated by B lymphopoiesis in the fetal liver and omentum switching to bone marrow after birth, the primary 'pre-immune' B-cell repertoire evolving during the first 2 months after birth in gut-associated lymphoid tissue (GALT) and the secondary 'immune' B-cell repertoire generated upon B-cell activation by immunogen binding.^{2,67} The neonatal B-cell repertoire starts developing between the second and third week of gestation.^{68,69} Interestingly, B lymphopoiesis is very limited in the bone marrow of adult rabbits,⁷⁰ indicating that B lymphopoiesis is mainly restricted to early development. However, B cells with germline antibody sequences were found in the adult spleen.⁷¹ In addition, in a rabbit-to-rabbit adoptive transfer model, it was shown that rabbit B cells were able to engraft into host stem cell niches,⁷² which led to the conclusion that rabbit B cells are long-lived and potentially self-renewing and may thus sustain the rabbit antibody repertoire throughout a rabbit's lifetime.⁷³

Rabbits mainly rearrange their heavy chains first followed by their light chains⁷⁴ and thus follow the primary B-cell development pathway proposed by Ehlich *et al.*⁷⁵ In brief, this

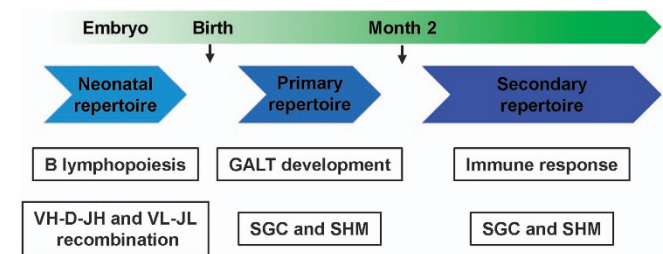


Figure 3 Ontogeny of rabbit B-cell and antibody repertoires. The three principally different developmental stages of B-cell and antibody diversification in the rabbit are shown. GALT; gut-associated lymphoid tissue; SGC, somatic gene conversion; SHM, somatic hypermutation.

model proposes the VH–D–JH recombination of the heavy chain, followed by its expression as a pre-B-cell receptor. Upon stimulation of the pre-B-cell receptor, VL–JL light-chain recombination is started. Some evidence for non-templated nucleotide-addition (also known as N-nucleotides) has been observed in rabbit V κ –J κ rearrangements in 1-day-old rabbits, suggesting that terminal deoxynucleotidyl transferase was recently expressed.⁷⁶

Although a potential pool of 200 VH genes are present for heavy-chain rearrangement,^{66,77} a majority of these are infrequently or not expressed.⁷⁸ There are 10–20 D genes and 4–5 JH genes.⁷⁹ However, it is known that certain genes from these clusters are preferentially utilized: VH1, the most D-proximal VH gene, is present in 80–90% of all heavy-chain gene rearrangements.^{68,79,80} Three different allotypes VHa1, VHa2 and VHa3 are known, resulting in the VHa-positive serotype.^{81,82} The remaining 10–20% of heavy chains contain VHx, VHy or VH z genes.^{83,84} This finding was somewhat surprising, because there is another functional, D-proximal VH sequence significantly closer to the VH1 segment. Indeed, it could be shown that the VH4 segment is utilized in 80–90% of the heavy-chain rearrangements in VH1-negative Alicia strain rabbits. However, this heavy-chain rearrangement was very rare in adult rabbits, suggesting that the VH4 rearrangement is non-productive in most cases and thus lost in the course of development.⁶⁹ Lanning *et al.*⁷³ suggested negative self-antigen effects and non-efficient pairing with light chains and surrogate light chains during B-cell development as possible reasons. In addition, preferential utilization of D2a, D2b and D1 as well as JH4 could be shown.⁷⁹

It is thought that rabbits compensate for this preferential utilization of certain VH, D and JH genes in part by N-nucleotide addition at the VH–D and D–JH junctions during recombination.^{68,69} Using high-throughput sequencing of antibody repertoires from three New Zealand White rabbits, Lavinder *et al.*⁸⁵ determined that the mean \pm s.d. length of the third complementarity-determining region (CDR) of the heavy chain, which is known as HCDR3, includes both junctions, and thus is the most hypervariable of all six CDRs, is 15 ± 4 (mode = 13) amino acids compared to mouse and

human HCDR3s with mean \pm s.d. lengths of 11 ± 2 (mode = 10 amino acids) and 15 ± 4 (mode = 15) amino acids, respectively. These findings confirmed that rabbit HCDR3s share more similarity with human HCDR3s than mouse HCDR3s and could be relevant for therapeutic applications.⁸⁶

Although there is limited heavy-chain usage in the neonatal rabbits' B-cell repertoire, there may be compensation by usage of diverse light chains. There are two types of rabbit light chains— κ and λ —with the κ light chain present in two different isotypes called K1 and K2.⁸⁷ Four K1 κ -light-chain allotypes—b4, b5, b6 and b9—have been observed in domesticated rabbits. Notably, the constant domain sequences of these rabbit κ -light-chain allotypes have much higher amino-acid sequence diversity than human Ig allotypes with pairwise amino-acid sequence differences of up to 40%.⁸⁸ This high degree of polymorphism exceeds the allotypic diversity of the three heavy-chain allotypes, a1, a2 and a3 (~20% amino-acid sequence differences), and even surpasses the allotypic diversity of major histocompatibility complex loci.¹ K1 is by far the most abundant isotype and represents between 70 and 90% of all light chains, whereas the rest is usually a mixture of both K2 and λ -light chains.⁸⁹ Many K1 light chains are characterized by an intrachain disulfide bridge between cysteine 80 in V κ and cysteine 171 in C κ (Figure 2).⁹⁰ This additional disulfide bridge, which was discovered biochemically^{91,92} and later confirmed by X-ray crystallography,^{93,94} is not found in human or mouse light chains. It may contribute to the stability of rabbit antibodies and is one of many peculiarities of Ig evolution in vertebrates. This disulfide bridge is absent from rabbits of the K1-negative Basilea mutant strain that only expresses K2 and λ -light chains. Some but not all of the K1 light chains of rabbits homozygous for the b9 κ -light-chain allotype⁸⁶ have a cysteine 108 in J κ that is thought to create an alternative intrachain disulfide bridge with cysteine 171 in C κ .^{89,90} These allotypes play an important role for the generation of rabbit mAbs by phage display and are discussed in more detail below. So far, ~50 functional V κ genes have been identified.^{76,95} Recently, using next-generation sequencing of rabbit antibody repertoires, Kodangattil *et al.*⁹⁶ demonstrated that light-chain rearrangements are significantly more diverse than heavy-chain rearrangements in rabbits. In addition, the imprecise junction of VL and JL genes in rabbits encompasses particularly long stretches of N-nucleotides, resulting in, on average, longer LCDR3s (12 ± 2 amino acids; mode = 12) compared to mouse and human (9 ± 1 amino acids; mode = 9; Figure 4).⁸⁵ The longer rabbit LCDR3s are occasionally stabilized by disulfide bridges.⁹⁷ Taken together, by generating a more diverse neonatal light-chain repertoire followed by a comparable degree of further diversification into primary and secondary light-chain repertoires, the light chain can compensate for the limited diversity of heavy-chain repertoires in rabbits. This may also explain the dominance of the rabbit light chain in general and LCDR3 in particular in several of the antibody/antigen complexes for which three-dimensional structures have been determined by X-ray crystallography.^{94,97–100}

The limited neonatal antibody repertoire is further diversified between week 4 and 8 after birth resulting in the primary antibody repertoire.⁶⁷ This postnatal diversification is an unusual phenomenon and has so far only been observed in rabbits and pigs.¹⁰¹ The two main mechanisms are somatic gene conversion (SGC; templated) and somatic hypermutation (SHM; non-templated; Figures 3 and 4). SGC, predominantly used only in chicken and rabbit Ig gene diversification,¹⁰² leads to the replacement of large nucleic acid sequence stretches with DNA fragments from non-utilized VH genes.^{80,103,104} High-throughput sequencing revealed a mean \pm s.d. tract lengths of 59 ± 36 nucleotides.⁸⁵ Thus, the non-expressible majority of VH genes play an important role for heavy-chain diversification. The predominance of this mechanism in rabbit (23%) compared to human (2.5%) and mouse (0.1%) heavy chains, as determined by high-throughput sequencing,⁸⁵ is thought to lead to decreased wastage: a substantial portion of VH–D–JH recombinations with N-nucleotides addition have out-of-frame junctions, whereas SGC, a homologous DNA recombination event,¹⁰⁵ tends to favor in-frame substitutions and extensions.⁸⁹ In addition, SHM is observed at considerable levels during this developmental stage.^{83,106,107} Following rearrangement in the neonatal B-cell repertoire, rabbit light-chain genes also undergo SGC and SHM to diversify the primary B-cell repertoire (Figures 3 and 4).¹⁰⁸ Further corroborating the importance of the light chain for generating diversity in rabbit antibody repertoires, SGC is more frequent (32%) and has a longer mean \pm s.d. tract length (86 ± 48 nucleotides) in rabbit κ -light chains compared to rabbit heavy chains.⁸⁵

Interestingly, the generation of the primary repertoire is highly GALT-dependent. In short, GALT involves the uptake of pathogens from the gastrointestinal track by specialized epithelial cells known as M cells and the presentation of antigens to B cells leading to B-cell stimulation, diversification and proliferation.¹⁰⁹ Surgical removal¹¹⁰ of GALT tissues, such as the Peyer's patches in the small intestines, the sacculus rotundus and the appendix¹¹¹ resulted in severely immunodeficient rabbits. In addition, it was observed that germ-free rabbits developed abnormal GALT.^{112,113} Lanning *et al.*¹¹⁴ demonstrated that this directly correlated with reduced somatic VH–D–JH diversification compared to germ-exposed rabbits.

The secondary B-cell repertoire is generated upon antigen-dependent B-cell stimulation. Again, additional diversity is introduced by SGC and SHM in both heavy and light chains (Figures 3 and 4).^{115,116} These events further broaden the B-cell repertoire directed against a certain set of antigens associated with a specific pathogen.⁷³

The discussed idiotypic and allotypic peculiarities of rabbit antibodies further extend to the isotype. Notably, rabbits only have one IgG isotype (one C γ gene) compared to four IgG isotypes in mice (IgG1, IgG2a, IgG2b and IgG3) and humans (IgG1, IgG2, IgG3 and IgG4). In contrast, rabbits have 13 C α genes giving rise to at least 10 functional IgA isotypes, the most diverse IgA system known,^{1,117} compared to just one

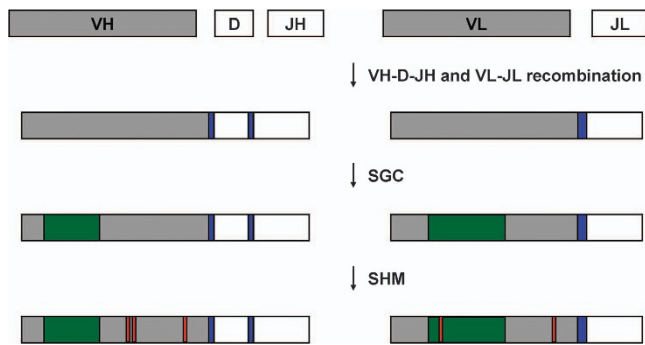


Figure 4 Molecular mechanisms of sequence diversification in rabbit antibody repertoires. Top: VH–D–JH and VL–CL recombination takes place in the neonatal B-cell repertoire. In the rabbit, N-nucleotide additions (blue) at VL–CL junctions (encoding LCDR3) are more extensive than those found at VH–D and D–JH junctions (encoding HCDR3). Middle: SGC events (green) diversify the VH and VL portion of rabbit heavy and light chains in primary and secondary B-cell repertoires and mostly localize to regions encoding HCDR1, HCDR2, LCDR1 and LCDR2. Bottom: SHM events (red) further diversify rabbit heavy and light chains in primary and secondary B-cell repertoires. SGC, somatic gene conversion; SHM, somatic hypermutation.

IgA isotype in mice and two in humans. Collectively, the unique features of B-cell ontogeny and antibody repertoire make rabbits a valuable source for the generation of antibodies of high affinity and specificity.

GENERATION OF RABBIT MABS

Hybridoma technology

The discovery and development of hybridoma technology for the generation of mAbs by Georges Köhler and César Milstein in 1975 has had a huge impact on biomedical research and its application to modern medicine.^{118,119} Hybridoma technology is a method to generate stable cell lines that constantly secrete a defined mAb. For this purpose, B cells derived from an immunized animal are fused with a myeloma cell line in the presence of polyethylene glycol. The hybridomas generated this way are cloned by limiting dilution, screened for favorable mAb characteristics and then expanded in culture to obtain high amounts of the desired mAb.¹¹⁹ Generally, two hybridoma types can be distinguished: first, homo-hybridomas where both host B cells and fusion cell line emerged from the same species and, second, hetero-hybridomas derived from two different species.¹²⁰

Ever since their initial discovery, multiple aspects of the general technique have been modified in order to avoid certain problems related to fusion efficiency, hybridoma stability and mAb titers.¹²¹ Hybridoma technology has been used extensively to generate thousands of different mAbs against a wide variety of antigens. In fact, the majority of FDA-approved chimeric, humanized and human mAbs originate from hybridoma technology.¹²⁰ However, all of these were derived from mouse B cells or transgenic mouse B cells with human Ig genes. Due to the favorable properties of rabbit antibodies, many

groups tried to develop methods for the generation of rabbit hybridomas. This endeavor was significantly complicated by the absence of rabbit myeloma cell lines.¹²² Viral transformation of rabbit B cells to generate myeloma-like cell lines also proved to be difficult and rather inefficient.¹²³ For these reasons, substantial efforts focused on generating rabbit–mouse hetero-hybridomas. Unfortunately, all hetero-hybridomas generated in the early days of hybridoma technology revealed poor fusion efficiency, genetic instability and impaired functional rabbit heavy- and light-chain pairings.^{124–126} In 1988, Raybould *et al.*¹²² generated the first stable rabbit–mouse hetero-hybridoma by polyethylene glycol-mediated fusion of rabbit spleen B cells with the mouse myeloma cell line SP2/0–Ag14. Even though they observed stable rabbit IgG expression for several months, other groups observed genetic instability and concomitant decrease of mAb secretion. These shortcomings could be partially addressed by extensive efforts to regularly subclone the rabbit–mouse hetero-hybridoma.¹²⁷ The first rabbit homo-hybridoma was developed in 1995 in the laboratory of Katherine Knight.¹²⁸ In order to obtain a potential rabbit fusion cell line, transgenic rabbits were generated by single-cell zygote microinjection and mated to generate v-abl/c-myc double transgenic rabbits. This method led to the discovery of the first stable rabbit plasmacytoma cell line, 240E-1,¹²⁹ which could be used as an efficient fusion partner to generate rabbit homo-hybridomas. Interestingly, Spieker-Polet *et al.* also showed in this publication that B cells derived from different rabbit tissues led to the generation of hybridomas secreting different ratios of IgG, IgM and IgA. However, the stability of the obtained homo-hybridomas was still a major concern and IgG secretion decreased over time. Although this decay is frequently also observed for mouse homo-hybridomas, it appeared to be more drastic in the case of rabbit homo-hybridomas.^{30,130} For this reason, Zhu and Pytela¹²¹ attempted to further improve the initial 240E-1 cell line by iterative subcloning to screen for clones with higher fusion efficiency, yielding hybridomas with higher genetic stability and more stable rabbit IgG secretion. The obtained fusion cell line 240E-W and its successors 240E-W2 and 240E-W3, which are characterized by higher fusion efficiency and the absence of endogenous rabbit heavy- and light-chain secretion (US Patent 7,429,487),⁴¹ are part of the proprietary RabMab platform of Epitomics, Inc. (now Abcam, Inc.) and were used to generate the therapeutic rabbit mAbs sevacizumab and APX005M discussed above.

Aside from therapeutic applications, rabbit mAbs generated by hybridoma technology have become highly valuable reagents for diagnostic applications and for laboratory research. For example, highly specific rabbit mAbs can detect activating mutations in the tyrosine kinase domain of EGFR by IHC of lung cancer tissues.¹³¹ Rabbit mAbs are also suitable to detect post-translational modifications.^{132,133} Further, rabbit mAbs against the HIV-1 protein gp120 were shown to mimic neutralizing human anti-HIV-1 gp120 mAbs, promoting rabbit immunization as model for HIV-1 vaccine development.^{94,134–136}

Phage display technology

Bacteriophage, also simply known as phage, are viruses that infect and replicate within bacteria. Phage display was invented by George Smith, who discovered that the minor coat protein (pIII) of filamentous phage can be modified at its amino terminus to present peptide sequences without affecting phage infectivity. In addition, these phage particles contained the genomic information encoding the respectively modified coat protein, thus physically linking genotype and phenotype. A combination of phage selection and amplification could be used to efficiently enrich phage that contained certain peptide sequences.¹³⁷ However, the incorporation of larger peptide sequences, protein domains or whole proteins represented a serious challenge due to decreasing infectivity. For this reason, two-component systems were developed that consisted of a phagemid encoding the pIII fusion protein and a helper phage contributing the phage genome to encode all proteins necessary for generating infectious phage particles. Usually, these helper phage contain a modified packaging signal, leading to the preferential assembly of phage particles containing the phagemid. In the early 1990s, the first filamentous phage display antibody libraries based on pIII fusion proteins were published using either scFv or Fab fragments.^{138,139} To date, these formats are still dominating the selection of mAbs by phage display.^{140–142}

Although phage display was established with mouse and human antibody libraries to mine immune, naive and synthetic antibody repertoires,^{120,141} the fact that rabbit mAbs were difficult to generate by hybridoma technology for a number of years provided a strong incentive for exploring the accessibility of rabbit immune antibody repertoires by phage display. The first rabbit antibody library selected by phage display was reported by Ridder *et al.*,¹⁴³ using a scFv format as in subsequent independent studies.^{39,40,144–151} Rabbit antibody libraries in Fab format followed in short succession.^{152,153} Due to the higher expression levels of human compared to rabbit constant domains in bacteria, a chimeric rabbit/human Fab format consisting of rabbit variable domains VL and VH recombinantly fused to human constant domains CL and CH1, respectively, proved particularly successful for the selection of rabbit mAbs by phage display^{19,154–160} and their subsequent humanization (Figure 5).¹⁵⁵ However, the above-discussed intrachain disulfide bridge between cysteine 80 and cysteine 171 found in rabbit κ light chains of the dominating K1 isotype posed a challenge to the chimeric rabbit/human Fab format as human CH1 does not harbor a cysteine 171. In fact, only few Fab originating from the K1 isotype were selected,^{86,155} indicating that the free thiol group of cysteine 80 is disfavored and that its presence diminishes the selectable diversity of chimeric rabbit/human Fab. Indeed, chimeric rabbit/human Fab derived by phage display from immunized K1-negative Basilea strain rabbits revealed higher sequence diversity and higher affinity compared to those from K1-positive New Zealand White strain rabbits immunized with the same immunogens.⁸⁶ Interestingly, the same study by Popkov *et al.* also compared rabbits homozygous for the

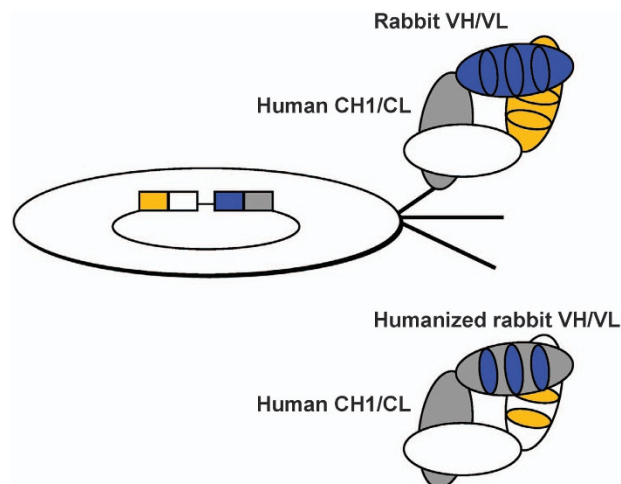


Figure 5 Chimeric rabbit/human Fab and humanized rabbit Fab. Top: in contrast to rabbit Fab, chimeric rabbit/human Fab, which consist of rabbit variable domains (blue and orange) and human constant domains (gray and white), are well expressed in *Escherichia coli* and displayed on phage. The phage particle shown displays one chimeric rabbit/human Fab copy linked to the phage surface by the C-terminal pIII protein domain as its phenotype and, as its genotype, contain the corresponding single-stranded phagemid that encodes the Fab. This physical linkage of phenotype and genotype is the essence of phage display. Bottom: following selection by phage display, chimeric rabbit/human Fab can be humanized, which typically involves the grafting of the six rabbit CDRs (blue and orange) into human framework regions (gray and white).

b9 κ -light-chain allotype (Figure 1) with the presumed alternative intrachain disulfide bridge between cysteine 108 and cysteine 171. Fusion of the rabbit V κ and human C κ encoding sequences in the generation of the chimeric rabbit/human Fab library removes cysteine 108, thus avoiding the exposure of a free thiol group. Consequently, immunized b9 allotype rabbits also revealed superior selectable diversity and were subsequently used in several additional studies.^{21,32,161} These include the generation of a chimeric rabbit/human Fab against the HIV-1 protein Rev.⁹⁹ The Fab was able to invert Rev polymerization and allowed for the formation of Fab-Rev co-crystals that could be analyzed with X-ray crystallography providing the first three-dimensional structure of Rev and the first three-dimensional structure of a rabbit mAb.⁹⁸ Notably, the crystal structure revealed a dominant role for LCDR3 in the antigen-binding site (Figure 6). A cyclic peptide derived from LCDR3 was shown to bind HIV-1 Rev with high affinity and to potently inhibit Rev polymerization,¹⁶² corroborating the functional importance of the above-discussed high sequence diversity of rabbit light chains.

A key advantage of using the natural Fab format for phage display is its robust monomeric nature that permits affinity-driven selections.¹⁴² By contrast, the unnatural scFv format has a tendency to dimerize, trimerize and tetramerize,^{163,164} potentially causing avidity-driven selections.¹⁴¹ Thus, we

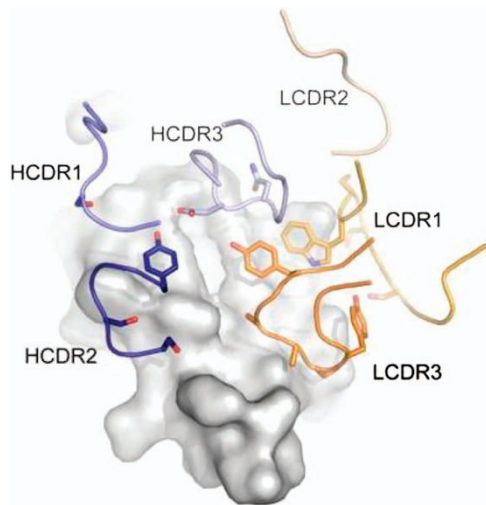


Figure 6 Crystal structure of a chimeric rabbit/human Fab selected by phage display in complex with HIV-1 Rev (PDB accession code 2X7L).^{98,99} The three CDR loops of the heavy chain are colored blue and the three CDR loops of the light chain are colored orange. Amino-acid residue side chains that interact with the antigen (gray) are drawn as sticks. Note the dominating contribution of LCDR3 to the paratope. When synthesized as cyclic peptide, LCDR3 on its own was able to bind HIV-1 Rev with high affinity.¹⁶²

recommend using chimeric rabbit/human Fab format-based phage display for applications that require rabbit mAbs of high affinity. Even if the application calls for scFv, such as for the generation of intrabodies, the chimeric rabbit/human Fab format has been used for selection by phage display followed by conversion to the rabbit scFv format.^{160,165–167} In addition to Fab and scFv formats, single-domain antibody formats, that is, VL or VH alone, which due to their smaller sizes have advantages for certain applications, such as the recognition of cryptic epitopes,¹⁶³ have also been engineered from rabbit mAbs.^{168–170} The noted dominance of the rabbit light chain may make phage display of rabbit VL libraries particularly attractive.

Alternative methods

As discussed, hybridoma technology and phage display have distinct virtues and shortcomings that have fueled the development of alternative techniques to generate mAbs. Unlike hybridoma technology, phage display relies on the proper transcription, translation, folding and assembly of light and heavy chains in bacteria. Also, a potential disadvantage of phage display, as well as other display technologies, is their combinatorial nature, leading to the random pairing of light and heavy chains.¹²⁰ As discussed above, limiting factors encountered during hybridoma technology, at least initially, included fusion efficiency and hybridoma stability.^{130,171} Also, unlike mouse hybridoma technology, rabbit hybridoma technology was patented, imposing intellectual property restrictions that have contributed to the incentive of developing alternative methods.

In the generation of human mAbs, prominent alternative methods include the clonal expansion of B cells by, for example, Epstein–Barr virus immortalization, and single B-cell sorting followed by light- and heavy-chain-encoding DNA amplification and sequencing.¹²⁰ Notably, like hybridoma technology but unlike phage display, both methods yield natural light- and heavy-chain pairs. Single B-cell isolation based on antigen capture by fluorescence-activated cell sorting, magnetic beads, solid-phase panning or hemolytic plaques followed by light- and heavy-chain cloning has also been applied to the generation of rabbit mAbs from peripheral B cells,^{172–176} which does not require killing of the animal and allows for multiple sampling points from primary and secondary lymphoid tissues.¹⁷⁷ Antigen capture-driven bulk spleen B-cell isolation from immunized rabbits followed by light- and heavy-chain-encoding DNA amplification and their combinatorial assembly and expression in scFv–Fc or IgG format in mammalian cells has also been reported.¹⁷⁸ In the fluorescent foci method, plasma cells from rabbit bone marrow are identified by fluorescent microscopy, isolated and subjected to light- and heavy-chain-encoding DNA amplification and sequencing.¹⁷⁹

High-throughput DNA sequencing technologies have been applied to the *in silico* generation of mAbs as well as to the analysis of naive and immune antibody repertoires in different species.¹⁸⁰ In the rabbit, high-throughput DNA sequencing of B-cell repertoires along with mass spectrometry analysis of bulk serum IgG has been used for the deconvolution of mAbs.^{64,181} Interestingly, this method revealed that the serum IgG response to an invertebrate protein following hyperimmunization was oligoclonal rather than polyclonal, comprising 34 rabbit mAbs belonging to 30 distinct clonotypes.⁶⁴

HUMANIZATION OF RABBIT MABS

The human immune system is intended to recognize and selectively remove potentially pathogenic organisms and substances. This is also true for nonhuman proteins, such as mouse and rabbit mAbs. The respective immune responses are characterized by high titers of human antibodies directed against these foreign antibody sequences.¹⁸² This has two main physiological consequences; first of all, it leads to side effects comparable to allergic reactions of different levels of severity and, second, it leads to the rapid elimination of the administered mAb, thus limiting its diagnostic or therapeutic efficacy.^{183,184} For these reasons, nonhuman mAbs are now routinely converted to chimeric mAbs by combining the nonhuman variable domains with human constant domains¹⁸⁵ and further to humanized mAbs by grafting all or some of the CDRs of the nonhuman variable domains into human frameworks.¹⁸⁶

The first chimeric and humanized rabbit mAbs were reported by Rader *et al.*¹⁵⁵ In this study, chimeric rabbit/human Fab selected by phage display were humanized by grafting the six rabbit CDRs, three from each light and heavy chain, into human frameworks that were diversified at certain positions to allow the selection of either human or rabbit

residues by phage display. The resulting humanized rabbit mAbs retained the high affinity and specificity of their parental mAbs. As discussed above, aside from having higher utility for downstream clinical applications, using chimeric rabbit/human Fab format for phage display has the added advantage of affording higher expression levels in bacteria and, concomitantly, higher display levels on phage.¹⁵⁵

Since this first report, a number of additional humanized rabbit mAbs have been published and patented. In general, humanization strategies that have worked for mouse mAbs also work for rabbit mAbs, whether it is by rational design,^{17,33,187} directed evolution¹⁸⁸ or a combination of both.^{155,189} As is the case for mouse mAbs, grafting of all six CDRs followed by iterative fine-tuning of framework residues is the most frequently used method for humanizing rabbit mAbs. Borrás *et al.* used a general acceptor framework for the humanization of rabbit scFv. In this study, a certain human framework carrying five specific human-to-rabbit mutations was able to generate humanized rabbit mAbs to two different antigens with conserved affinities and specificities, and improved biophysical properties.²⁴ Humanization strategies that confine the content of parental rabbit antibody sequences only to the most hypervariable of the CDRs, such as LCDR3 and HCDR3,¹⁹⁰ have also been successfully applied to rabbit mAbs.¹⁸⁸

Thus far, there have been no reports that analyze the immunogenicity of clinically investigated humanized rabbit mAbs. Recent studies indicate that patients can still mount an immune response to humanized or even fully human mAbs. These findings indicate that additional aspects, such as idiotype, allotype, glycosylation and aggregation, contribute to the immunogenicity of mAbs.¹⁹¹ Nonetheless, the occurrence of these unwanted side effects is significantly lower than previously observed for nonhuman antibodies.¹⁸²

SUMMARY AND OUTLOOK

Driven by the success of rabbit pAbs on one hand and mouse mAbs on the other hand, rabbit mAbs have become highly successful reagents for laboratory research and for diagnostic and therapeutic applications. The unique ontogeny of rabbit B cells affords highly distinctive antibody repertoires rich in *in vivo* pruned binders of high diversity, affinity and specificity. The ability to access rabbit antibody repertoires by hybridoma technology, phage display and alternative methods has fueled the increased use of rabbit mAbs in many different applications. Rabbit mAbs generated by hybridoma technology are particularly attractive for IHC, with many studies demonstrating higher sensitivity compared to benchmark mouse mAbs. Rabbit mAbs generated by phage display have found the use for applications ranging from the detection of uranium in water⁴⁰ to chimeric antigen receptor T cell therapy of cancer.²² Given that humanization strategies are now well established, the therapeutic utility of rabbit mAbs is especially intriguing. With the first rabbit mAb-derived therapeutics already in clinical trials, we predict that rabbit mAbs will gain further traction in preclinical and clinical pipelines over the next decade.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr HaJeung Park for preparing Figure 6, and Drs Rose G. Mage and Weimin Zhu for reading and editing the manuscript.

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