

Progress and perspectives of rabbit monoclonal antibodies

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ABSTRACT

Since the advent of murine hybridomas, the emergence of a variety of monoclonal antibody (mAb) technologies has enabled the wide applications of murine monoclonal antibodies in medicine, life science, agronomy, and food science. Compared with murine monoclonal antibodies, rabbit monoclonal antibodies (RabmAbs) exhibit higher affinity, presenting with increased detection sensitivity and greater specificity for the particular structure of epitopes. This paper reviews the history, preparation techniques, advantages and disadvantages, current applications, and future perspectives for RabmAbs.

Keywords: rabbit monoclonal antibody, hybridoma technology, genetically engineered antibody technology, phage display technology, single B-cell technology

INTRODUCTION

In 1975, Köhler and Milstein outlined their proposal for a murine-derived hybridoma technique, which marked the beginning of the widespread development of monoclonal antibody (mAb) technologies *in vitro*^[1]. Currently, murine mAbs can be utilized for diagnosing clinical diseases, detecting food safety issues, identifying pesticide residues, and so on^[2–4]. However, murine mAbs are prone to human anti-murine antibody reactions when used in clinical settings, resulting in reduced antibody potency. Therefore, in addition to the further development of new murine mAb technologies, researchers have been actively searching for model animals without the drawbacks of murine mAbs for several years.

In 1988, to develop a rabbit myeloma cell line, Raybould *et al.* obtained the first rabbit-murine heterozygous hybrid tumor by polyethylene glycol-mediated fusion of rabbit splenic B cells with the

murine myeloma cell line SP2/0-Ag14^[5]. However, there still remained problems related to genetic instability and the inability to achieve long-term antibody secretion. In 1995, Spieker-Polet *et al.* successfully isolated the rabbit plasmacytoma cell line 240E-1 from c-myc/v-abl transgenic rabbits, and developed the first rabbit-rabbit homologous hybridoma^[6]. Yam and Knight continued their work and found that the cell line encountered two main problems: on one hand, the hybridoma gradually lost its ability to secrete antibodies after iterative subcloning; on the other hand, the cell line itself expressed endogenous rabbit immunoglobulin G (IgG), IgM, and IgA^[7].

In 1997, Zhu *et al.* solved the problems of genetic instability and endogenous rabbit IgG secretion by improving the culture medium and iterative subcloning to obtain a more stable 240E-W with higher secretion^[8]. In 2001, Zhu *et al.* further improved the fusion cell line and derived cell lines

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240E-W2 and 240-W3. Extensive fusion experiments demonstrated that the absence of endogenous rabbit's heavy and light chain secretion was related to higher fusion efficiency. It was possible to prepare rabbit monoclonal antibodies (RabmAbs) with stable performance by hybridoma technologies. The international patent was obtained in 2013^[9]. This paper reviews the history, preparation techniques, advantages and disadvantages, current applications, and future perspectives for RabmAbs.

ADVANTAGES OF RABBIT MONOCLONAL ANTIBODIES

Murine is the first choice for most monoclonal antibody preparations owing to its ease of handling, convenient sourcing, and low cost. However, there is a growing trend towards the use of RabmAbs. The immune system of rabbits differs from that of rodents, and therefore, different mechanisms are utilized to produce diverse and high affinity antibodies^[10]. In general, compared with murine mAbs, RabmAbs have the following advantages.

Improved recognition specificity

As the development of rabbit B-cells differs from that of humans and mice, their B-cell antibody affinity maturation contains both somatic high-frequency mutations and somatic gene replacement mechanisms, whereas mice only possess somatic high-frequency mutations^[11]. Therefore, RabmAbs have the potential of higher specificity and a broader range of antigenic epitopes. Mehta *et al.* generated RabmAbs to be specific to amyloid- β 1-37 (A β 37) as it did not react with A β 36, A β 38, A β 39, A β 40, and A β 42 in an enzyme linked immunosorbent (ELISA) or immunoblotting^[12]. Furthermore, Mehta *et al.* prepared RabmAbs specific for pE3-A β in 2018, and they showed that RabmAb had no reactivity with A β 16, A β 40, A β 42, A β 3-11, and pE11-17 A β peptides in ELISA^[13]. This indicated that Mehta's series of research contributed to supporting the diagnosis of patients with probable Alzheimer's disease.

Broader antigenic epitope repertoire

Antibody affinity reflects the ability of an antibody molecule to react with a semi-antigen molecule or determinant cluster of antigen molecules. The magnitude of antibody affinity can be expressed in terms of the dissociation constant (KD) (molality/litre). Due to the unique immunoaffinity maturation of rabbits, it has been found that the affinity of RabmAbs is 10–100 times higher than that

of murine mAbs, and their KD can reach the picomolar level ($KD=10^{-12}$ mol/L)^[14]. Taking this into account, the development of a more sensitive RabmAb assay has great potential for application. Li *et al.* discovered RmAb1, a rabbit monoclonal antibody, to ractopamine (RAC) with a high affinity of 0.007 ng/mL. Moreover, RmAb1 for RAC were 0.0042–0.014 μ g/L on indirect competitive enzyme-linked immunosorbent assay. It showed that RmAb1 contributed to accurate screening of RAC residue in animal urine^[15].

More identified epitopes

Compared with mice, rabbits have a more comprehensive lipid and glycolipid antigen presentation, which enables rabbit antibodies to possess a broader spectrum of antigen binding sites^[16]. Bystryn *et al.* compared melanoma-associated antigens recognized by both murine mAbs and rabbit polyclonal antibodies^[17]. The results showed that antigens that are immunogenic in humans may not necessarily be immunogenic in mice but can be recognized in rabbits. Pan *et al.* used a RabmAb to identify a novel epitope antigen, 10A37 of the third variable loop (V3) of HIV-1 gp120^[18]. Their results revealed that the novel V3 binding mode targeted the C-terminal side of the V3 crown, and its epitope structure was similar to that of the epitope bound to human antibodies. Therefore, it can be used as a potential vaccine target, making the development of an HIV-1 vaccine possible.

Rare antigen recognition epitopes tend to be more valuable antibody druggability-associated epitopes. In addition, gene conversion and somatic hypermutation phenomena lead to more mutations in the rabbit antibody repertoire. For this reason, against the same immunogen, the richness of epitopes for rabbit antibodies is much greater than that for murine antibodies. Rabbit V regions seem to accumulate two-third more mutations, when compared with humans and mice, perhaps helping to diversify the repertoire of antibodies generated by a limited number of germline genes, which produces a more diverse and complex immune response to target antigens and specifically recognizes certain antigenic epitopes, such as phosphorylated peptides, carbohydrates, and glycans^[19–22]. The above characteristics of RabmAbs will facilitate the exploration of the drug action mechanism.

Longer half-life

A rabbit mAb has a longer half-life compared with the murine mAb. Lewis *et al.* reported hRabMab1, a

humanized rabbit mAb specific for human alternatively spliced tissue factor (asTF). HRabMab1 has a long half-life (about 5 weeks) in circulation and binds asTF with a KD in the picomolar range. Given that hRabMab1 has a favorable character, it is a promising candidate for pancreatic ductal adenocarcinoma clinical treatment^[23]. In addition, in terms of immunotherapeutic agents, the murine antibody has a short half-life in serum and induces the production of human anti-murine antibodies. In contrast, rabbit mAbs show lower immunogenicity while retaining high specificity and affinity for human antigens^[24].

Simpler immunoglobulin structures

Human and murine immunoglobulins can be classified into five classes, namely IgA, IgD, IgM, IgE, and IgG, with several subclasses of IgG (**Table 1**)^[8]. Unlike murine and human IgG, rabbit IgG has only one isotype, with fewer amino acid residues at the N-terminal and D-E loops, and disulfide bonds in the variable region of the heavy chain, as shown in **Fig. 1**^[25]. Researchers have speculated that disulfide bonds contribute to the stability of the antibody^[25–26]. In addition, there are few rabbit IgG light chains: 90%–95% of the light chains are Cκ1 (isotype κ1), while only 5%–10% are λ-type^[27]. Rabbit-derived antibodies are easily engineered and manipulated, which adds more possibilities for antibody drug development.

Table 1 Immunoglobulin (Ig) subclasses of different species^[8]

Species	IgG subclasses
Human	IgG1, IgG2, IgG3, IgG4
Mouse	IgG1, IgG2a, IgG2b, IgG2c, IgG3
Rat	IgG1, IgG2a, IgG2b, IgG2c
Rabbit	Only one IgG isotype
Chicken	IgY (comparable to IgG)

Generation of more hybridoma cells

Rabbits have larger spleens than murine species, and the number of fused B lymphocytes is approximately 50 times larger than that of mice, which allows the fusion assay to yield several times more hybridoma cells. That, in combination with high-throughput screening techniques, makes it possible to produce more antibodies^[28–29]. In addition, compared with murine hybridomas, rabbit hybridoma cells are more suitable for high-density culture with longer screening time, shorter intervals between cell passages, and greater experimental plasticity. Zhu *et al.* accidentally discovered rabbit hybridoma features

through experimental error, and thus founded Epitomics^[8–9]. Based on these studies, rabbit hybridomas can be easily and efficiently screened for antibody drugs *in vitro*.

RABBIT MONOCLONAL ANTIBODY PREPARATION TECHNIQUES

The main techniques commonly used for the preparation of RabmAbs include hybridoma technique, phage display technique, and single B-cell technique.

Hybridoma technology

Many types of antigens including DNA, peptides, proteins, whole cells, and tissues, are used to raise RabmAbs. Rabbits mount significant immune responses to small molecules and haptens, but rodents do not. Therefore, peptides and proteins are the most commonly used immunogens. Hybridoma technique relies on the fusion of rabbit myeloma cells (240E-W2) with antigen-immunized rabbit splenocytes or plasma cells to obtain antibody-secreting and indefinitely proliferating hybridoma cells, which are then screened to obtain a specific cell line that can be cultured to produce a large number of biologically active rabbit-derived monoclonal antibodies.

In contrast to mouse hybridomas, rabbit-rabbit hybridomas are similarly unstable, have relatively low fusion efficiency, are time consuming, and have patent limitations. For use in hybridoma cell culture, an increasing number of commercial hybridoma feeder additive (HFA) has replaced the peritoneal macrophage feeder layer. Furthermore, it was observed that recombinant human interleukin 6 (IL-6) had an effect on rabbit hybridomas that increased the immunoglobulin yield^[30].

Phage display technology

Since the *in vitro* antibody screening technique of phage antibody display was developed by Gregory P. Winter and John McCafferty in 1985, it has become the most widely used and well-established technique for *in vitro* antibody screening. The principle of this technique is based on the phage's ability to insert the B-cell antibody gene sequence from immunized rabbits into the structural gene of the phage capsid protein, allowing the antibody to form a fusion protein with the phage capsid protein, which is then assembled and displayed on the surface of the phage as the daughter phage is amplified, ensuring the relative spatial structure and biological activity of the antibody^[31–32].

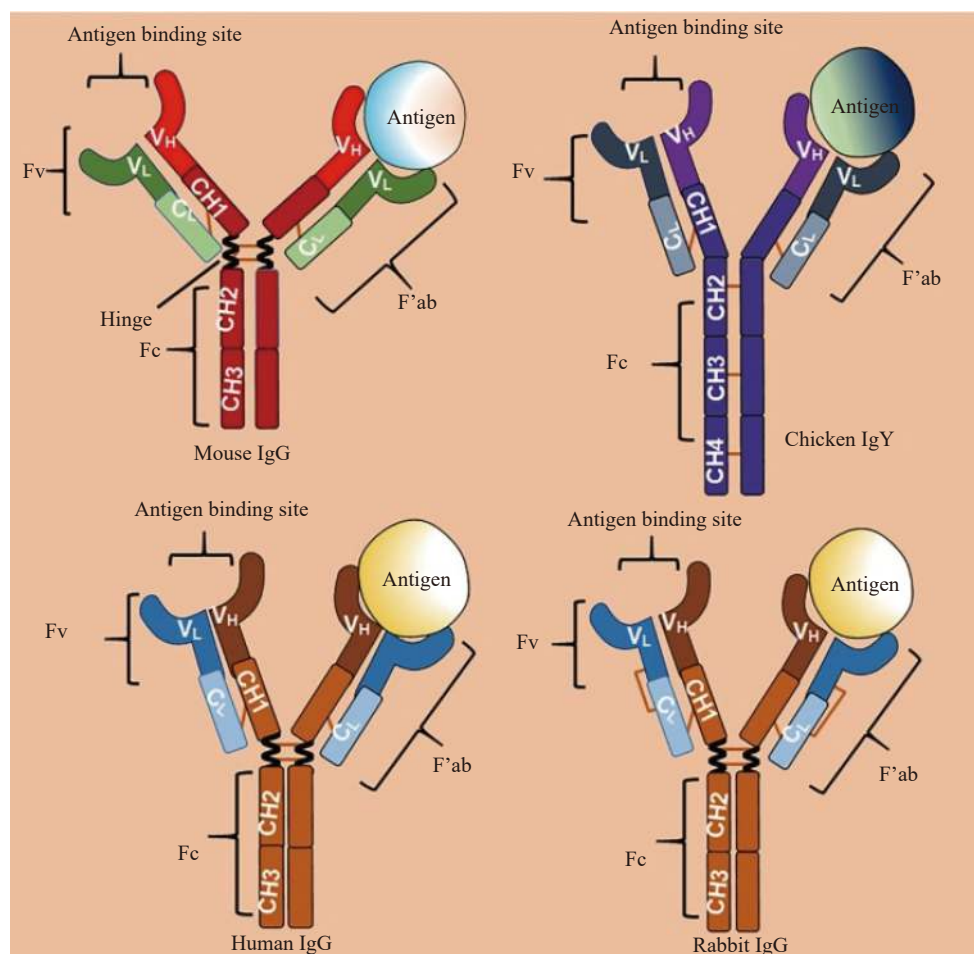


Fig. 1 Schematic drawing of natural rabbit, mouse, chicken, and human IgG^[25]. Generally, a 150-kDa IgG comprises of two identical κ or λ light chains paired with two identical heavy chains. The light chain consists of an N-terminal variable domain (VL), followed by one constant domain (CL). The heavy chain consists of an N-terminal variable domain (VH), followed by three constant domains (CH1, CH2, and CH3) generally; however, the heavy chain of avian IgY contains four constant regions (CH1, CH2, CH3, and CH4). Schematic drawing of the structure of natural rabbit antibody IgG. The 150-kDa rabbit IgG molecule contains two identical light chains (blue) paired with two identical heavy chains (brown). The light chain consists of an N-terminal variable domain (VL), shown with its three complementarity-determining regions (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 disulphide 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 approximately 90% IgG- κ (K1), approximately 10% IgG- κ (K2), and <1% IgG- λ antibodies.

Compared with the more restricted hybridoma technique, phage display technology has the following main advantages^[33–37]: (1) it can easily screen potential target antibodies on a large scale; (2) the antigenic epitope or conformational modification of existing antibodies can reduce immunogenicity, and improve affinity and stability; (3) the rapid proliferation of prokaryotic cells and the low cost of culture make it easy to prepare high-purity antibodies in large quantities for large-scale research and application.

An increasing number of researchers have used this

technology to develop many promising RabmAbs. For example, Aguiar *et al.* used phage display technology and next generation sequencing (NGS) to develop rabbit-derived single-domain antibodies which can cross the blood-brain barrier^[38]. Davies *et al.* developed a blood marker antibody repertoire for inflammasome activation based on rabbit phage technology^[39].

However, the technique has several limitations^[40–41]: (1) the obtained antibodies' affinity is poor, so a complex antibody process is required. However, the *in*

vitro modification of the antibodies is still a technical bottleneck to be addressed; (2) compared with hybridoma technology, the screening process for phage antibody libraries is more cumbersome, and the positive clone rate is not significantly higher; (3) this technique is more technically demanding than hybridomas; (4) the phage antibody display process must go through bacterial transformation and phage packaging, which means the heavy and light chains of antibodies may not be properly transcribed, translated, post translationally modified, folded, and assembled.

In addition to phage display technology, there are also yeast, ribosome, bacterial, and mammalian cell surface displays^[36,42–43]. These rabbit-derived display technologies need to be further developed and optimized, which will enrich the possibility of preparing RabmAbs.

Single B-cell technology

Hybridoma technology has low cell fusion efficiency, requires sacrificing animals, has low stability of the obtained hybridoma cell lines, and faces patent protection barriers, while phage display technology causes the loss of natural cognate pairing of heavy and light chains^[44]. To overcome these issues, single B cell antibody technology has been recently developed.

This technology consists of the following short steps^[16,45]: (1) identification and isolation of specific single B cells; (2) single-cell amplification of Ig genes and cloning of Ig genes into expression vector; (3) expression of Ig genes in bacterial systems (for example *Escherichia coli*) or mammalian cell systems, such as human embryonic kidney 293 (HEK 293), Chinese Hamster Ovary (CHO) cells, and purification of proteins evaluated with Flow, ELISA, Western blot (WB), immunohistochemistry (IHC), *etc.*

The isolation of rabbit lymphocytes is limited to lack of useful surface markers. Hence, to select antigen-specific rabbit B cells for mAb expression, several new methods have been developed, including fluorescence activated cell sorting (FACS)^[46], fluorescence and micromanipulation^[47], lymphocyte panning^[48], HybriFree technology^[49], two-color antigen staining method^[50], and chip-based immunospot array^[51].

For B cell enrichment, there have been several relatively successful approaches in the industry: (1) flow cytometric sorting by Thermo Fisher, US, *etc.*^[52]; (2) magnetic bead sorting by Miltenyi Biotec, Germany, *etc.*^[53]; (3) the photo localization system based on Beacon[®] and Opto[®] Memory B discovery

rabbit workflow developed by Berkeley Light, US^[54]; (4) microfluidic technology represented by Cytomine, Sphere Fluidics, UK^[55]; (5) high-throughput single B cell screening technology based on BEAM-Ab from 10× genomics by Signalway Antibody Company, US^[56]; (6) the SMab[™] platform, an optimized formulation used to culture sufficient amounts of B cells by Yurogen Biosystems LLC, China^[57].

Another important step is the gene encoding the heavy and light chains of the corresponding mAbs from a single B cell, mainly involving the following approaches: single cell RT-PCR linked *in vitro* expression (SICREX)^[58], NG-XMT[™] technology by Cell Signaling Technology^[59], and Ecobody technology^[60].

In general, the use of the above methods to obtain mAbs presents several disadvantages for commercial applications: (1) higher technical barriers, higher experimental costs, and strict environmental requirements; (2) there is an increased chance of high cycle number or multiple rounds of PCR, which mediates mutations in the target genes with the potential for loss of functional antibodies; (3) antibody genes are cloned from single cells and verified by *in vitro* recombinant expression, which is labor-intensive and time-consuming. Therefore, these methods are not suitable for large-throughput screening.

CURRENT STATUS OF RABBIT MONOCLONAL ANTIBODY APPLICATIONS

RabmAb technology has been widely used in basic medical research, disease diagnosis, blood testing, and other disciplines. With the continuous development of mAb technology over the past 20 years, RabmAbs have emerged as highly sensitive, specific, and accurate products, which are fast and easy to be used in several fields, including medical diagnosis, food testing, pesticide residue analysis, crop protection, *etc.*

Medical diagnosis and treatment

Owing to the high specificity and affinity of RabmAbs, the U.S. Food and Drug Administration (FDA) has approved a number of RabmAbs for clinical diagnostic applications. The relevant information is shown in [Table 2](#)^[61]. These mAbs can help reduce the misdiagnosis rate of disease through pathological testing and improve patient survival rate, demonstrating the high clinical application potential of RabmAbs. For example, through the utilization of single B-cell technology, rabbit mAb 9E1 prepared by

Hong *et al.* can recognize linear epitope of the receptor binding structural domain (RBD) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein. 9E1 can specifically recognize the highly conserved linear epitope within 12 amino acids on the RBD ⁴⁸⁰CNGVEGFNCYFP⁴⁹¹. The 9E1

antibody was used in IHC detection on SARS-CoV-2 infected tissue sections and in WB for viral S proteins with good results compared with polyclonal antibodies^[62]. The research results suggest that 9E1 can be used as a useful tool for pathological and functional studies of SARS-CoV-2.

Table 2 FDA approved rabbit monoclonal antibodies for clinical diagnostic applications^[55]

Authorized company	Antigen	How to use	Range of application	Approval time
Ventana	Human Ki-67	IHC	Adjuvant diagnosis of cervical cancer and primary HPV screening	2020
Ventana	Human PD-L1 (SP263)	IHC	Diagnosis of invasive uroepithelial carcinoma	2017
Ventana	Human PD-L1 (SP142)	IHC	Diagnosis of invasive uroepithelial carcinoma	2016
Dako	Human PD-L1	IHC	Pathological diagnosis of non-squamous non-small cell lung cancer (NSCLC) and melanoma	2016
Agilent	Human PD-L1	IHC	Diagnosis of NSCLC	2015
Ventana	Human estrogen receptor	IHC	An aid in the management, prognosis, and prediction of hormone therapy for breast carcinoma	2012
Ventana	Human estrogen receptor	IHC	Diagnosis of breast cancer hormone therapy	2011
Ventana	<i>Helicobacter Pylori</i> (<i>H. pylori</i>)	IHC	Diagnosis of <i>H. pylori</i> infection	2011
Dako	Human estrogen receptor	IHC	Diagnosis of breast cancer hormone therapy	2009
Lab Vision	Human estrogen receptor	IHC	Diagnosis of breast cancer hormone therapy	2006
Lab Vision	Human progesterone receptor	IHC	Diagnosis of breast cancer hormone therapy	2006
Ventana	Human C-kit/CD117	IHC	Pathological diagnosis of gastrointestinal mesenchymal tumor	2004
Ventana	Human HER2	IHC	Patients with pathologically diagnosed metastatic breast cancer treated with Herceptin	2000

PD-L1: programmed cell death-ligand 1; C-kit/CD117: stem cell growth factor receptor; HER2; human epidermal growth factor receptor 2; IHC: immunohistochemistry; HPV: human papillomavirus; NSCLC: non-small cell lung cancer. Retrieved from www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm (Devices@FDA) for "rabbit monoclonal antibody".

The latest cancer data released by the World Health Organization's International Agency for Research on Cancer (IARC) 2020 showed that the number of worldwide cancer cases may be expected to be as high as 28.4 million in 2040, indicating a 47% increase compared to 2020^[63]. Therapeutic antibody drugs that specifically bind to tumor targets have become an important component of biopharmaceuticals with significant efficacy and are promising prospects for disease treatment. To avoid the immune response of RabmAbs in humans, however, they should be humanized before treatment. Currently, the U.S. FDA has approved some applications for therapeutic rabbit-derived monoclonal antibodies. The corresponding antibody-drug information is shown in [Table 3](#).

Food safety inspection

RabmAbs are widely used in the detection of toxic substances in foodstuffs such as dairy, meat, and brewing products. Since RabmAbs with high affinity can specifically target trace antigens, it is possible to determine the content of pathogenic microorganisms and toxins in food with high sensitivity and high

specificity. Furthermore, for easy and fast operation, they can be widely used in safety control and quality management of food production and scientific research. Liu *et al.* prepared a new RabmAb against sulfonamides (SAs) by rabbit hybridoma technique, and established a sensitive competitive indirect ELISA method based on the novel RabmAb for the rapid detection of sulfonamides in milk^[64].

Pesticide residue analysis

Pesticide immunoassay techniques using antibodies as analytical tools are specific, sensitive, convenient, fast, and economical. These techniques have been developed with the advancement of antibody preparation technology, and play an important role in pesticide residue analysis. Onder *et al.* used a single B-cell cloning to produce rabbit mAb 1C6 against tyrosine-modified diethoxyphosphotyrosine (depY). Rabbit 1C6 showed a 10-fold higher affinity for the depY peptide compared with murine mAb 3B9. In addition, human neuroblastoma SH-SY5Y cells and mouse neuroblastoma N2a cells incubated with a subcytotoxic dose of 10 μ mol/L chlorpyrifos oxon

Table 3 Information of rabbit-derived monoclonal antibodies in selected clinical trials

Authorized company	Targeted protein	Drug	Clinical number approved by FDA	Progress	Indication	Approval/Update time
Novartis	VEGF (scFv)	Brolucizumab	NCT02307682 NCT02434328	Listed	Treatment of neovascular age-related macular degeneration	2019
Alder/Lundbeck	CGRP	Eptinezumab	NCT04152083 NCT02974153	Listed	Migraine prevention	2021
Alder	IL-6	Clazakizumab	NCT03744910	Phase 3	Desensitization of therapeutic solid organ transplant recipients	2022
Apexigen	CD40	APX005M	NCT03123783	Phase 2	Treatment of non-small cell lung cancer and metastatic melanoma	2021
YooYoung Pharmaceutical	HGF	YYB101	NCT02499224	Phase 1	Treatment of advanced solid tumors	2021
3SBio/Apexigen	TNF alpha	-	NCT02460393	Phase 1	Treatment of rheumatoid arthritis	2017
Simcere/Apexigen	VEGF	Sevacizumab	NCT02453464	Phase 2	Treatment of metastatic colorectal cancer	2016

VEGF: vascular endothelial growth factor; scFv: single chain antibody fragment; CGRP: calcitonin-gene-related peptide; IL-6: interleukin-6; CD40: tumor necrosis factor receptor superfamily, member 5; HGF: hepatocyte growth factor; TNF: tumor necrosis factor. Retrieved from the U.S. FDA clinical trial information search website: <https://clinicaltrials.gov/>

contained depY-modified proteins detected by 1C6 on WB. It was shown that the rabbit 1C6 antibody would be helpful neurotoxicity due to long-term low-dose exposure to organophosphorus pesticides^[65].

Crop protection

RabmAbs have great research value and application significance in the diagnosis of crop pathogens and viruses, which have the potential to improve the diagnosis of crop bacteria, viruses and other diseases in order to take timely control measures and thus reduce economic losses. Miyoshi *et al.* used RabmAbs prepared from citrus mosaic virus capsid proteins, which showed high sensitivity for ELISA detection of citrus mosaic virus in leaf extracts with high potential for commercialized development^[66].

CONCLUSIONS AND FUTURE PERSPECTIVES

After decades of development from restricted hybridoma technology to phage display and single B cell technology, the more effective RabmAbs were developed in combination with high-throughput methods. With highly attractive advantages, RabmAbs have emerged as a nonnegligible force in life science and related fields. It is reasonable to believe that with the rapid development of science and technology, RabmAb preparation technology will become increasingly mature, and it is expected that more RabmAbs will be introduced to clinics in future,

which means that the opportunities and challenges facing RabmAbs are unprecedented.

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