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REVIEW

Monoclonal antibodies: Principles and applications of immmunodiagnosis and immunotherapy for hepatitis C virus

Ashraf Tabll, Aymn T Abbas, Sherif El-Kafrawy, Ahmed Wahid

Ashraf Tabll, Microbial Biotechnology Department (Biomedical Technology Group), Genetic Engineering and Biotechnology Division, National Research Centre, Dokki 12622, Egypt

Aymn T Abbas, Biotechnology Research Laboratories, Gastro-eneterology Surgery Centre, Mansoura University, Mansoura 35516, Egypt

Aymn T Abbas, Sherif El-Kafrawy, Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah 22254, Saudi Arabia

Sherif El-Kafrawy, National Liver Institute, Menoufia University, Shebin El-Kom 32511, Egypt

Ahmed Wahid, Biochemistry Department, Molecular and Cellular Biology Unit, Faculty of Pharmacy, Minia University, Minya 11432, Egypt

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Correspondence to: Ashraf Tabll, PhD, Head, Microbial Biotechnology Department (Biomedical Technology Group), Genetic Engineering and Biotechnology Division, National Research Centre, El Behoose Street, Dokki 12622, Egypt. ashraf.tabll@yale.edu
Telephone: +20-23-3362609
Fax: +20-23-330931

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Abstract

Hepatitis C virus (HCV) is a major health problem worldwide. Early detection of the infection will help better management of the infected cases. The monoclonal antibodies (mAb) of mice are predominantly used for the immunodiagnosis of several viral, bacterial, and parasitic antigens. Serological detection of HCV antigens and antibodies provide simple and rapid methods of detection but lack sensitivity specially in the window phase between the infection and antibody development. Human mAb are used in the immunotherapy of several blood malignancies, such as lymphoma and leukemia, as well as for autoimmune diseases. In this review article, we will discuss methods of mouse and human monoclonal antibody production. We will demonstrate the role of mouse mAb in the detection of HCV antigens as rapid and sensitive immunodiagnostic assays for the detection of HCV, which is a major health problem throughout the world, particularly in Egypt. We will discuss the value of HCV-neutralizing antibodies and their roles in the immunotherapy of HCV infections and in HCV vaccine development. We will also discuss the different mechanisms by which the virus escape the effect of neutralizing mAb. Finally, we will discuss available and new trends to produce antibodies, such as egg yolk-based antibodies (IgY), production in transgenic plants, and the synthetic antibody mimics approach.

Key words: Hepatitis C virus; Monoclonal antibodies; Immunodiagnosis; Immunotherapy

Core tip: The monoclonal antibodies (mAb) of mice are predominantly used for the immunodiagnosis of several viral, bacterial, and parasitic antigens. Human mAb are used in the immunotherapy of several blood malignancies, such as lymphoma and leukemia, as well as for autoimmune diseases. In this review, we discuss methods of mouse and human monoclonal antibody production. We will demonstrate the role of mouse mAb in the detection of hepatitis C virus (HCV) antigens as rapid and sensitive immunodiagnostic assays. We will also discuss the role of HCV-neutralizing antibodies in the immunotherapy of HCV infections and in HCV vaccine development.

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INTRODUCTION

Monoclonal antibodies (mAb or moAb) are monospecific antibodies that have the ability to bind to the same epitope[1]. These antibodies are made by homogeneous hybrid cells (B cells) that are each clones of the same origin parent cell. Polyclonal antibodies, on the other hand, are made of several different immune cells (B cells). Hybridomas are hybrid cell lines that are generated through the fusion of an antibody-producing B cell with a myeloma (B cell cancer) cell. Myeloma cells are characterized by the ability to grow in tissue cultures and the absence of antibody chain synthesis. All antibodies produced by these hybrid cells (using hybridoma technology) are of a single specificity, and therefore mAb. The establishment of cell lines producing mAb was first reported by Köhler *et al*[2] in 1975.

The hepatitis C virus (HCV) is a major health problem worldwide. According to the estimates of the World Health Organization, this virus infects more than 180 million people across the world (representing 2%-3% of the world's total population)[3,4]. HCV (genotype 4) is one of the major health issues in Egypt, infecting 22% of the country's general population[3,5-7]. HCV is a small-enveloped, single-stranded RNA virus belonging to the family Flaviviridae. The genome encodes a single polyprotein that is co- and post-translationally processed into four structural and six non-structural proteins[4,8]. This is done by different cellular- and viral-encoded proteases. The envelope glycoproteins E1 and E2 are two structural proteins located on the surface of the HCV, and hence play a crucial role in HCV entry into hepatocytes. Their presence on the surface of the virus makes these two proteins, particularly E2, a major target for HCV antibody neutralization and interaction with host cellular receptors[3,8].

PRODUCTION OF MOUSE MAB

Mouse mAb by hybridoma technology

In 1975, Köhler *et al*[2] developed a hybridoma method for the production of mAb. The persistence of antibody-producing cells *via* their fusion with tumor cells may be an obvious procedure today, but at the time, this procedure was regarded as a key innovation that would allow for the unlimited yield of a specific antibody molecule. In 1984, Köhler *et al*[2] were awarded the Nobel Prize in Physiology or Medicine "for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of mAb. While mAb are now long-established as vital research products, their therapeutic use requires further development, particularly in terms of the humanization of mouse antibodies and recombinant productivity protocols. Several hundreds of mAb are currently under evaluation for the treatment of a broad range of conditions and use within a variety of therapeutics on the market[9]. The principle production of mouse mAb by hybridoma is shown in Figure 1. The different types and applications of mAb as diagnostic and therapeutic applications are presented in the Figure 2.

Hybridoma cell production has conventionally been performed *via* cell fusion between spleen cells (B cell source) and myeloma cell lines by chemical fusion techniques using for example polyethylene glycol (PEG). A recent publication by Kandušer *et al*[10] in 2014, however, describes another technique for cell fusion based on electrofusion. This technique is superior to the PEG method due to its high fusion efficiency. Kato *et al*[11] have stated yet another technique that involves CpG oligodeoxynucleotide (CpG ODN) for cell activation prior to electrofusion. Kato *et al*[11] reported that CpG ODN stimulation not only increases fusion efficiency but also the number of antibody-producing cells, leading to an increased number of positive clones obtained.

Rat and rabbit mAb can be produced by the hybridoma technology using rat and rabbit spleen cells, respectively. A recent study[12] generated rat hybridoma clones *via* the cell fusion of immunized rat spleen cells with mouse myeloma SP2/0 cells and screened the generated antibodies using recombinant mouse CXCL4 and rhCXCL4. This study concluded that the CXCL4 signaling pathway is a potential therapeutic target in numerous diseases including cancer. In addition, Zhang *et al*[13] used rabbit hybridoma to produce highly sensitive rabbit mAb targeting an emerging cell surface in mesothelioma and other solid tumors (Mesothelin). They concluded that the generated rabbit mAb may be promising candidates for monitoring and treating mesothelioma and other mesothelin-expressing cancers.

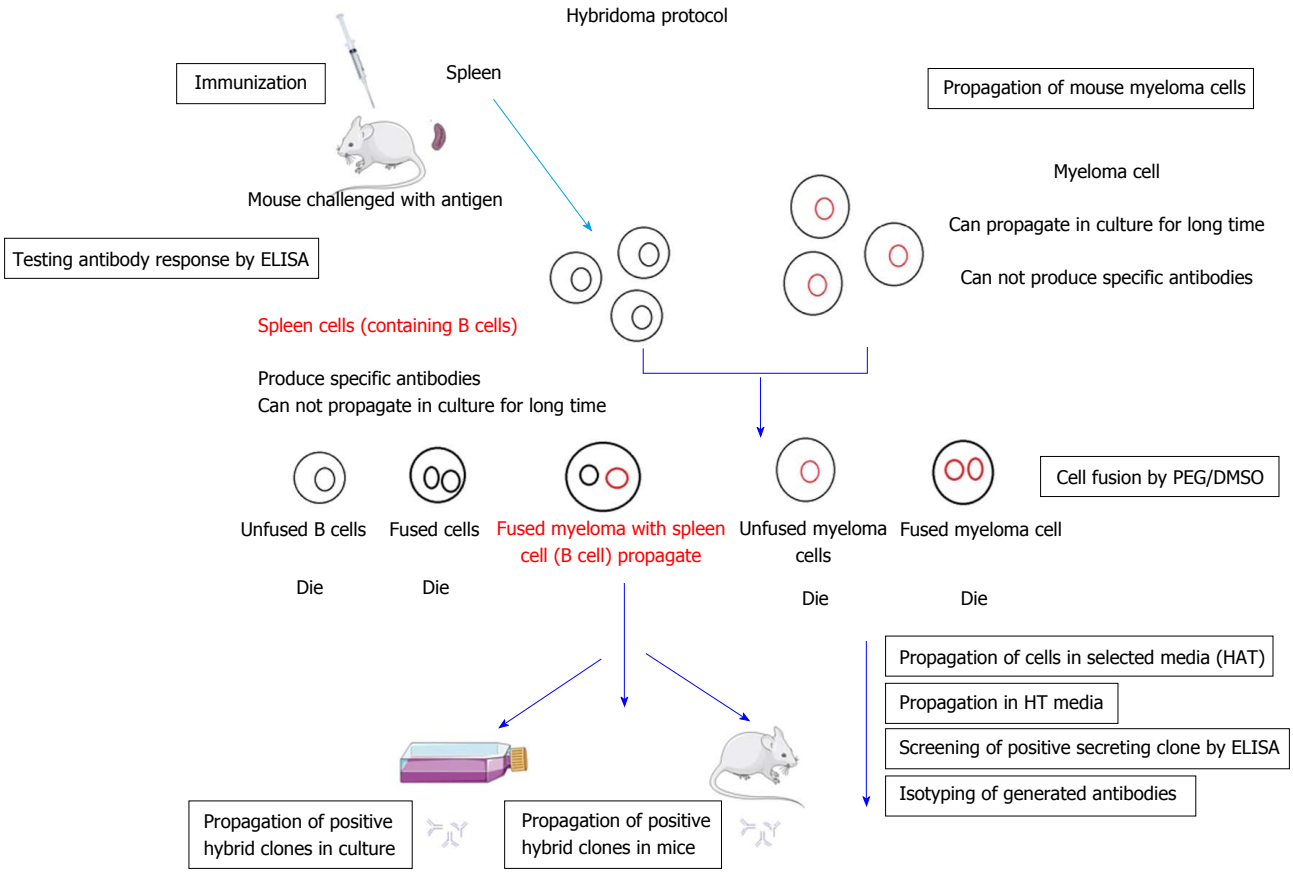

Figure 1 Diagrammatic procedure of the production of mouse monoclonal antibodies by hybridoma technology. ELISA: Enzyme-linked immunosorbent assay; PEG: Polyethylene glycol; DMSO: Dimethyl sulfoxide; HAT: Hypoxanthine-aminopterin-thymidine; HT: Hypoxanthine thymidine.

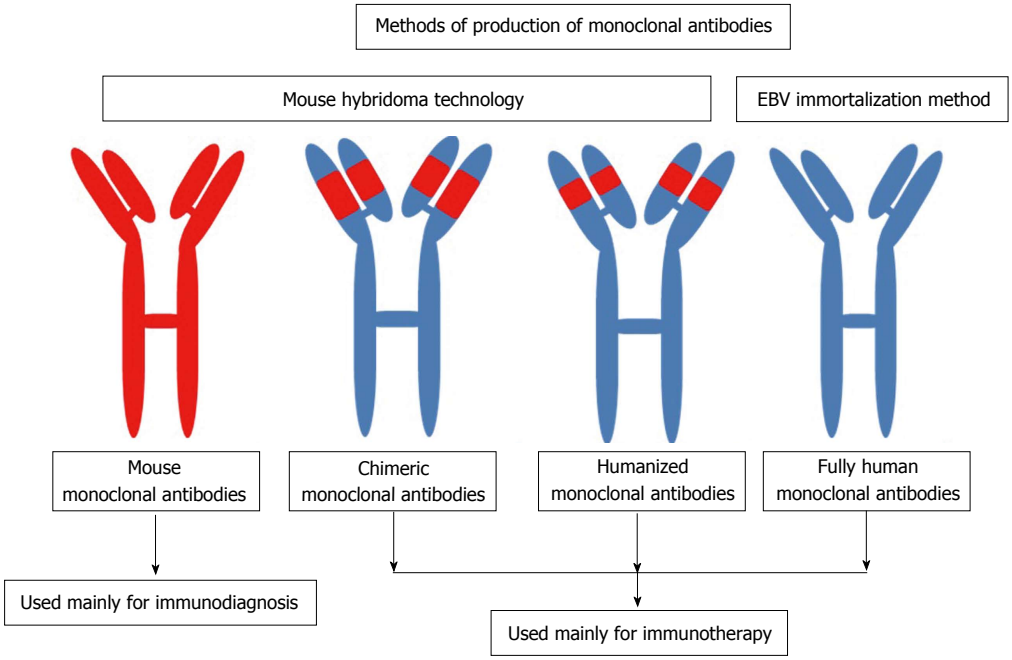

Figure 2 Diagrammatic presentations showing the applications of mouse and human monoclonal antibodies and their methods of production. EBV: Epstein-Barr virus.

PRODUCTION OF FULLY HUMAN MAB

There are several methods for the production of human mAb, such as phase display, transgenic mice, humanized mouse mAb, and immortalization by Epstein-Barr virus (EBV). In this review, we focus on the production

of fully human mAb by EBV immortalization. Human mAb (hMAb) provide novel ways for probing the B cell repertoire of various health and disease issues. Several difficulties have been encountered in the development of the hMAb, including cell line instability, low levels of specific antibody secretion, and poor cloning potency, particularly when using lymphoblastoid cells[14]. Martin *et al*[15] reported that the immortalization of B lymphocytes by EBV is a time consuming methodology for antibody production. EBV infects B cells *via* their CD21 receptors, which then transforms the B cells into lymphoblastoid cell lines that produce antibodies, representing the humoral immune response *in vivo*. Based on the type of parent cell, the generated antibodies target either an infective agent or a tumor cell, which makes them suitable therapeutic candidates against these diseases.

Compared to other antibody manufacturing techniques, the immortalization of B cells stands as the best technique, as the generation of fully human antibodies from the immortalized human B lymphocyte repertoire does not require immunization[16,17]. The first B lymphocyte immortalization experiment was performed by culturing B cells in the presence of a herpes virus obtained from a marmoset lymphocyte cell line B95-8[18,19]. Numerous changes to this procedure have since been tried, yet the immortalization and B lymphocyte rates remain inefficient. Several successful mAbs have been generated with this technique for use against different pathogens; Schieffelin *et al*[20] report that human mAb against dengue virus envelope were generated by the EBV transformation of B cells from patients after two years of naturally-acquired dengue virus infection. These antibodies were found to have completely different cross-reactivity, and neutralizing patterns. Schieffelin *et al*[20] and others[21,22] have used CpG2006 as stimulators for EBV immortalization. Furthermore, antibodies neutralizing the SARS corona virus and cytomegalovirus have been successfully created *via* the introduction of the polyclonal B lymphocyte activator CpG2006 into the B lymphocyte immortalization method and by B lymphocyte activation before EBV infection, respectively[21,22]. Moreover, Fraussen *et al*[23] report that seeding low B lymphocyte numbers per well serves to limit bias toward the advantageous outgrowth of fast-growing immortalized B cells including interleukin-2 (IL2) and CpG 2006.

Siemoneit *et al*[24] used herpes virus immortalization for the production of human organism antibodies to target HCV core macromolecules. In doing this, they revealed the establishment of two vegetative cell lines secreting human mAb to the 22-kD nucleocapsid macromolecule (core, p22) of HCV. Siemoneit *et al*[24] isolated B lymphocytes from an anti-HCV-positive donor and immortalized them by EBV infection. Two of the cell colonies were fused with the (mouse/human) heteromyeloma cell line K6H6/B5. The generated fused hybridomas produced antibodies of the IgG1/kappa (U1/F10) and the IgM/kappa (Ul/F11) isotype and were found to specifically react with the recombinant core macromolecule p22. Recently, Steinitz[25] reported that EBV has the *in vitro* ability to immortalize nearly all human B lymphocytes, which allows for the isolation of monoclonal cell lines that secrete specific human mAb.

The method of immortalization by EBV allows for the production of human mAb of different classes (IgM, IgG, IgA, and IgE) from any individual. Human mAb produced by EBV immortalization resemble the supplies of antibody molecules derived from the lymphocytes of blood donors. Therefore, Steinitz[25] concluded that human mAb are promising reagents of passive immunization for several diseases, such as cancer, and viral or bacterial infections. In our recently published paper[26], we aimed to produce human cell lines by manufacturing neutralizing human mAb for use against the envelope E1/E2 macromolecule of HCV. For this, we used two methods for the EBV immortalization of $CD22^+$ cells taken from HCV-positive patients: (1) immortalization with 100% EBV only; and (2) immortalization by 30% EBV and CPG2006 with IL2 (Figure 3). Our results indicated that cell stimulators play a role in the production of antibodies, with immortalization by 100% EBV only producing large clones compared to immortalization by 30% EBV and CpG2006 with IL2. The antibody levels, as measured by enzyme-linked immunosorbent assay (ELISA), showed high optical density in cells immortalized with 30% EBV and stimulants CpG2006 and IL2. Our results also indicated that the immortalization of low B cell numbers by 30% EBV, CPG2006, and IL2 was introduced with high efficiency and reproducibility leading to immediate generation of single clones that produced mAb. The specificity of the generated mAbs was confirmed by screening them against linear synthetic peptides (as epitopes) derived from HCV E1 (a.a 315-323) and two synthetic peptides derived from HCV E2 (a.a 412-419 and a.a 517-531) using in-house, ELISA-based optimized assays, all of which were studied by several investigators[27-29]. Fifteen clones secreting human immunoglobulin G against HCV E1/E2 protein were isolated. The ELISA results showed that one antibody was binding to the E2 peptide (a.a 517-531), while two antibodies were binding to the HCV E2 peptide (a.a 412-419). The three generated antibodies (IgG3, one antibody, and IgG2, two antibodies) showed high neutralization activity against HCVpp. We therefore concluded that these antibodies may be useful for the passive immunotherapy of HCV infections, particularly for HCV-positive liver transplantation patients.

USING MOUSE MAB FOR THE DETECTION OF HCV ANTIGEN(S) AS DIAGNOSTIC MARKERS

The detection of anti-HCV antibodies involves a simple, inexpensive, and quick test, yet this test has a low sensitivity in the first six to eight weeks of infection, or given the presence of several clinical conditions, such as chronic immunosuppression or hemodialysis[30]. A recent study[31] has shown that the proteins of Core and

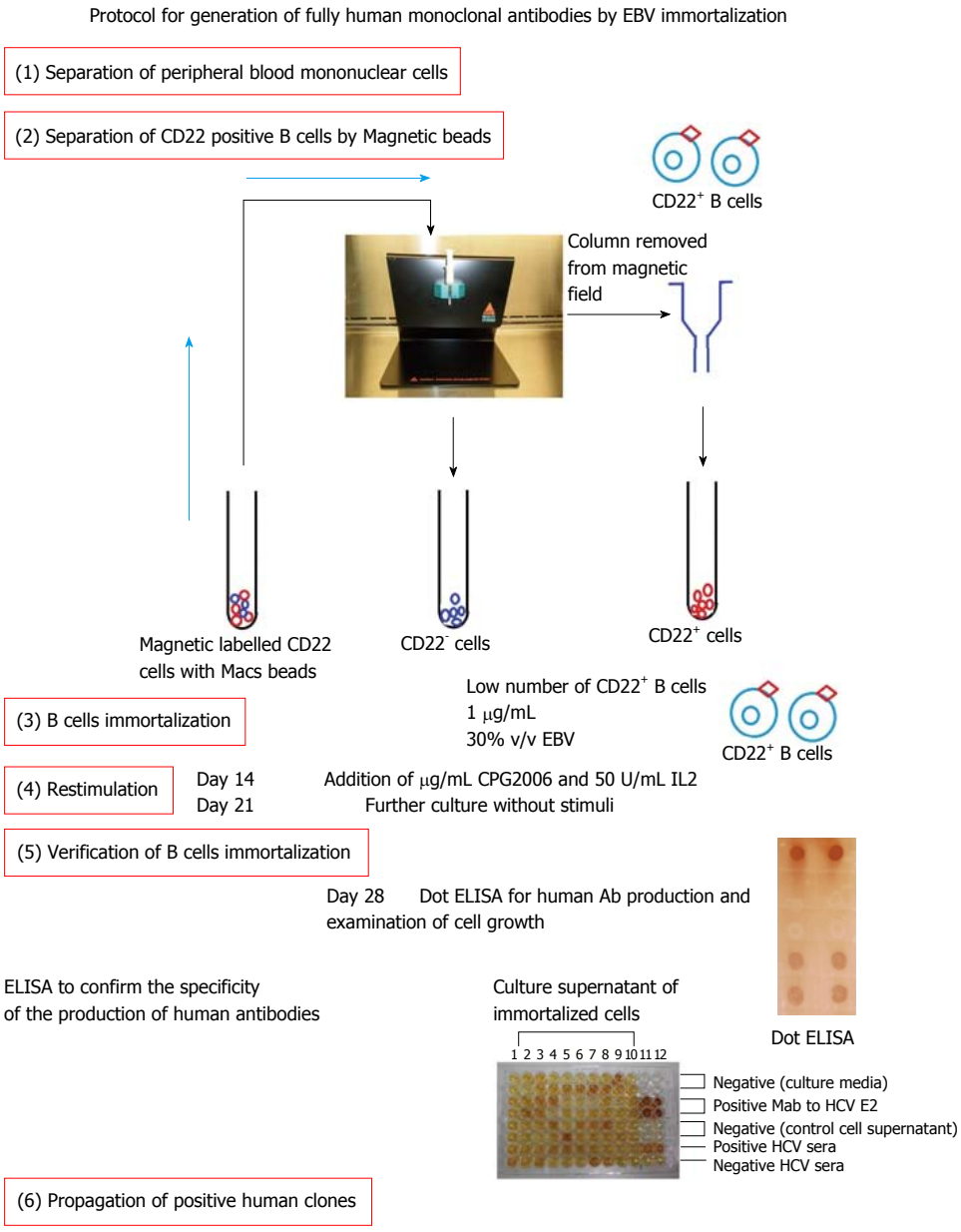

Figure 3 Generation of fully human monoclonal antibodies by Epstein-Barr virus immortalization. EBV: Epstein-Barr virus; HCV: Hepatitis C virus; IL2: Interleukin-2; ELISA: Enzyme-linked immunosorbent assay.

E2 genes within the antigenic regions of a local HCV-3a genotype can be used to develop highly sensitive, specific, and economical diagnostic assays for the detection of HCV infection. The recombinant antigen showed 100% reactivity against HCV-infected sera, with no cross reactivity in the HCV-negative sera. The authors therefore concluded that a mixture of Core and E2 antigens is potentially valuable for HCV-Ab detection[31].

The disadvantage of HCV diagnosis *via* the detection of HCV-Abs is the inability to distinguish between active and past infection. Given this, HCV-Ag detection is preferable, especially during the window phase of HCV infection. Recently, Florea *et al*[32] investigated the diagnosis of an HCV infection based on a HCV core Ag detection assay and found both a good correlation between the core Ag results with the HCV RNA viral load and very high specificity. Furthermore, Chevaliez *et al*[33] evaluated the clinical performance of the Architect HCV-Ag assay in terms of the detection and quantification of HCV core antigens in patients with chronic HCV genotype 1-6 infections. They concluded that the Architect HCV-Ag assay is highly specific, easy to perform, and represents a valuable screening, diagnostic, and monitoring tool.

Several investigators have demonstrated evidence of HCV antigens in liver tissue[34-37] serum samples[38,39] and plasma samples[40]. We reported the detection of HCV NS4 antigen in the sera of infected HCV patients using the Dot ELISA technique as a rapid assay[41]. The assay developed was able to detect the HCV target antigen in 95% of the serum samples from HCV-infected individuals with a specificity of 97% compared to the sera of uninfected individuals, using reverse transcription

polymerase chain reaction results as a reference. This antigen-detection-based method showed high positive predictive values (99%) and negative predictive values (90%). The added advantage of the assay is that it was able to detect HCV target antigens in the sera of patients during the window phase (negative for HCV-Ab and positive for HCV-RNA), as well as in the sera of both low and high HCV-RNA viral loads. The authors showed that their developed assay was highly sensitive and specific for HCV antigen detection, and that it could be applied for the mass screening of HCV infection.

In two reports[41,42], Attallah *et al*[41] describe their use of ELISA for the detection of HCV-NS4 and assessing the diagnostic performance of the assay in 883 chronic HCV patients. Taking quantitative HCV-RNA as a gold standard for HCV diagnosis, areas under the receiver operating characteristics (ROC) curves (AUC) were used to assess the diagnostic accuracy of ELISA for HCV-NS4. Attallah *et al*[42] identified HCV-NS4 to be at 27 kDa using Western blot. The areas under the ROC curves (AUC) in HCV-NS4 detection were 0.95 among patients with different pathological states of liver disease, with 0.93 for liver fibrosis, 0.95 for liver cirrhosis, and 0.98 for hepatocellular carcinoma (HCC). The mean ± SD (ng/mL) of HCV-NS4 in liver fibrosis was 94.2 ± 55.6, in liver cirrhosis was 99.3 ± 64.8, and in HCC was 124.9 ± 70.3. The authors therefore concluded that HCV-NS4 antigen detection using ELISA is a reliable test to confirm HCV infection. We established a hybrid cell line that produced mouse mAb targeting HCV E1 a.a 315-323[43]. We also produced mouse mAb targeting HCV E1/E2 and used them in the ELISA as a diagnostic assay for HCV infection (unpublished data). Our results showed a sensitivity of 80% and a specificity 96%.

HCV neutralizing antibodies

HCV cellular receptors are targets for HCV neutralization: Many HCV neutralizing antibodies target E1 and E2 HCV glycoproteins. However, an alternative strategy to preventing HCV entry may be achieved by targeting host receptors, such as CD81 and SR-BI. These antibodies block viral-host receptor interaction. A large number of broad spectrum, anti-HCV, host-targeting antivirals (HTAs) have been developed that trigger the innate immune system. Examples of these anti-HCV compounds include anti-SR-BI and toll-like receptor agonists[3,8]. The mechanism of action in this group of compounds involves the inhibition of certain cellular factors that are crucial to the HCV lifecycle. One of the main advantages of HTAs is that they act on host factors that are of a much lower rate of mutation[3]. Therefore, we are going to discuss primarily cellular receptors required for the attachment of HCV together with their involvement in the neutralization process.

CD81 receptor: The tetraspanin CD81 (26 kDa) is an integral membrane unglycosylated protein. CD81 is reported to possess several functions, such as signal transduction, cell activation, and cell adhesion. Moreover, in prior studies uding HCVpp and HCVcc systems, it has been confirmed that the CD81 receptor plays a major role in HCV cell entry[4]. A large number of broadly anti-HCV neutralizing antibodies block CD81 interaction with the HCV envelope glycoprotein E2. Indeed, it has been previously shown that anti-CD81 mAb inhibit the entry of both HCVcc and HCVpp into the Huh-7 cell line[44,45]. Resolving the crystal structure of CD81 complexed with E2 protein[44,45] has revealed that CD81 binds the HCV envelope glycoprotein E2 within certain specific amino acid residues (*i.e.,* 412-423, 432-447, 480-493, 528-535, and 544-551). K04, a recently generated anti-human CD81 monoclonal antibody, was shown to have a broad-spectrum antiviral action in the prevention and treatment of HCV infection[46].

Lipoprotein receptor scavenger receptor BI: The scavenger receptor class B type Ⅰ [scavenger receptor BI (SR-BI)] is highly expressed in hepatocytes. This receptor functions as a lipoprotein receptor that mediates cholesteryl ester selective uptake from high density lipoproteins[47]. The SR-BI receptor can bind both high density lipoproteins (HDL) and low density lipoproteins[8,47]. SR-BI has been previously shown to mediate interactions of E2-CD81. It has been suggested[45,48,49] that the SR-BI receptor interacts with HCV glycoprotein E2 hypervariable region 1 [hypervariable region-1 (HVR1), the first 27 amino acids in E2]. In line with this hypothesis, HVR1 deletion has been shown to inhibit E2-SR-BI interaction and to reduce HCV infectivity[50-52]. Indeed, it has been shown that HVR1 facilitates the interaction between HDL and SR-BI, which inhibits the neutralization of both HCVpp and HCVcc. HCV infection in cell cultures has also been shown to be inhibited with antibodies against SR-BI, confirming the crucial role of SR-BI in HCV cell entry[47,53,54]. Lastly, it was recently indicated that anti-SR-BI antibodies inhibit the HCV infection of different genotypes, both in cell cultures and humanized mice[55].

Other HCV receptors: Aside from SR-BI and CD81, other receptors including claudin-1 (CLDN1) and occludin (OCLN) compose the tight junction factors[56]. The tight junction multiprotein complex is comprised of four types of transmembrane proteins: Claudins, occludins, junction associated molecules, and the coxsackie virus B adenovirus receptors[57-59]. It is still unclear how the CLDN1 and OCLN inhibit HCV cell entry. Anti-claudin-1 antibodies neutralize HCV infectivity *via* inhibiting the interaction between CD81 and claudin-1 receptors, which is important to the viral entry process[58,59]. Occludin has been reported to co-precipitate with the HCV E2 glycoprotein. However, unlike CD81 and SR-BI and claudin-1 cellular receptors, no virus-specific neutralizing antibodies for occludin have been identified thus far[60].

HCV-NEUTRALIZING EPITOPES AND ANTIBODIES

The identification of the various mechanisms involved in immune protection is an important step in designing an HCV vaccine. The production of neutralizing antibodies by adaptive immune systems following vaccination has been prior demonstrated as a key strategy for protection against human viruses[61]. In the case of the HCV, a viral infection can persist even in the presence of a broadly neutralizing antibody, in many cases leading to chronic infection[44,62]; this can result in liver fibrosis, cirrhosis, and even eventually lead to hepatocellular carcinoma, causing death.

Anti-HCV antibodies can be targeted against structural and nonstructural protein epitopes (classified as either linear or conformational)[63,64]. The envelope glycoproteins E1 and E2 are considered major targets for neutralizing antibodies, as they are present on the surface of the HCV virus. Consequently, the development of an HCVpp system using unmodified HCV E1 and E2 envelope glycoproteins has allowed researchers to achieve remarkable progress in the study of neutralizing antibodies. However, little is understood so far as to the structure of the HCV envelope glycoproteins and how they interact with neutralizing antibodies[65].

Despite the fact that the HCV E1 glycoprotein is more conserved than E2, it has been proposed that E1 is of lower immunogenicity and hence a more difficult target for neutralizing antibodies[47,66]. However, two mAb against HCV glycoprotein E1 (IGH 505 and IGH 526) have been identified[27]. Both antibodies neutralize HCVpp bearing the E1 envelope glycoprotein of genotypes such as 1a, 1b, 4a, 5a, and 6a. These antibodies work within the region of E1 amino acids comprised of amino acids 313 to 327[27]. In addition to these two antibodies, the H-111 antibody has been reported to neutralize expressed E1 proteins from genotypes 1a, 1b, 2b, and 3a *via* binding to the 192YEVRNVSGVYH211 region of E1[67,68].

Anti-E2 human conformational-dependent HCV antibodies targeting E2 in HCVpp cell culture systems have been used to identify E2 epitopes. Due to such studies, three different regions of the E2 HCV glycoprotein E2 have been identified[50]. These regions include the E2 HVR1, the E2 HVR2, and the CD81 binding region of E2 and the C-terminus of HVR1[69]. The HCV glycoprotein HVR1 is a major target of neutralizing antibodies. This region is crucial for the virus, as it plays an important role in the HCV virus binding and entry process[50,70]. The physicochemical properties of the residues of HVR1 and its conformation are both highly conserved among various species despite the sequence variability of HVR1. It has been suggested that the sequence variability of E2 HVR1 is driven by the antibody selection of immune-escape variants[71]. Stable HVR1 sequences associated with resolved infection have also been reported, with HVR1 sequence change being suggested as one of the reasons for persistent HCV infection. This suggests that the most important target of antibody response to the HCV E2 glycoprotein is HVR1 (Table 1 for other examples)[72].

EXAMPLES OF HCV-NEUTRALIZING ANTIBODIES

Epitopes can be classified into two main groups (linear or conformational epitopes). Various viral epitopes that are targeted by neutralizing antibodies have been identified and characterized. Two human mAb, HCV1 and 95-2, have been identified as successfully neutralizing HCVpp belonging to different genotypes (*i.e.,* 1a, 1b, 2b, 3a, and 4a). In addition, a highly conserved linear epitope in E2 (amino acids 412 to 423) has been reported as recognized by HCV1 and 95-2 mAb[65]. Moreover, e137, a human monoclonal Fab, has been shown to bind to the HCV E2 glycoproteins of all HCV genotypes, with the exception of HCV genotype 5. Furthermore, it has been confirmed that this epitope interacts with highly conserved residues in all HCV genotypes, such as T416, W529, and D535[73]. CBH-5 has also shown itself to be capable of neutralizing all examined genotypes (genotypes 1-6). It was revealed that two of the amino acids comprising the epitope of CBH-5 are crucial for E2-CD81 interaction, which suggests direct competition between CBH-5 and CD81 to bind with the HCV E2 glycoprotein[74]. AP33 is another broadly neutralizing mouse monoclonal antibody that has been shown to neutralize all genotypes (*i.e.*, 1a, 1b, 2a, 2b, 3a, 4, 5, and 6). This antibody recognizes a highly conserved epitope in HCV glycoprotein E2 (amino acids 412 to 423)[75]. The high conservation of its epitope may have resulted in the broadly neutralizing activity of this antibody. Finally, AR3B displays a broadly neutralizing human antibody activity. AR3B neutralizing antibodies have been shown to protect against viremia in an infected mouse model[76].

NEUTRALIZING ANTIBODIES' MECHANISMS OF ACTION

The lack of cell culture-based assays to measure and quantify HCV activity has long hindered the study of the role of neutralizing antibodies in HCV infections. The mechanism of action in the antibody neutralization process still remains unclear. Several mechanisms through which neutralizing antibodies interfere with different stages of the HCV life cycle have been suggested[77,78], including immune aggregation and the blocking the attachment of the virion to the viral receptor which inhibits HCV infection. In addition to these mechanisms, neutralizing antibodies have been reported to interfere with other stages of the HCV life cycle following the binding process, such as the penetration of the virus through the cell membrane *via* host entry factors. Neutralizing antibodies may also prevent conformational changes important for the fusion of the virus to a host cell[77,78].

Table 1 Hepatitis C virus neutralizing antibodies

Neutralizing antibody	Epitope	Specificity	Escape mutants	Ref.
IGH505	E1 (313-326) linear	Cross-reactive	NA	[27]
IGH526	E1 (313-326) linear	Cross-reactive	NA	[27]
H-111	E1 (192-202) linear	Cross-reactive	NA	[68]
95-2	E2 (412-423) linear	Cross-reactive	NA	[65]
HCV-1	E2 (412-423) linear	Cross-reactive	NA	[65]
HC-1	E2 (523-540) conformational	Cross-reactive	No escape	[69,125]
3/11	E2 (412-423) linear	Cross-reactive	N415Y, N415D, N417S, G418D	[75]
3C7	E2 (396-407)	H strain	NA	[126]
AP33	E2 (412-423) linear	Cross-reactive	N415Y, N415D, N417S, G418D	[75]
CBH7	E2 conformational	Cross-reactive	NA	[74]
CBH5	E2 (523-540) conformational	Cross-reactive	NA	[68,127]
CBH2	E2 (425-447), E2 (523-540) conformational	Cross-reactive	D431G, A439E	[68,125,128]
CBH-8C	E2 conformational	Cross-reactive	NA	[129]
CBH-8E	E2 conformational	Cross-reactive	NA	[129]
CBH-11	E2 conformational	Cross-reactive	NA	[129]
CBH-17	E2 conformational	Cross-reactive	NA	[129]
CBH4B	E2 conformational	Cross-reactive	NA	[129]
CBH4D	E2 conformational	Cross-reactive	NA	[129]
CBH4G	E2 conformational	Cross-reactive	NA	[129]
9/27	E2 conformational	H strain	NA	[130]
A8	E2 (523-540) conformational	Cross-reactive	NA	[127]
J6.36	E2 Partially conformational	J6 strain	NA	[131]
HC-11	E2 (425-447) E2 (523-540) conformational	NA	L438F	[69,125]
Fab e20	E2 (523-540) conformational	Cross-reactive	NA	[132]
Fab e137	E2 (412-423) E2 (523-540) conformational	Cross-reactive	NA	[73]
1:7	E2 (523-540) conformational	Cross-reactive	NA	[127]
AR3A	E2 (394-424), E2 (437-447) E2 (523-540) conformational	Cross-reactive	NA	[76]
AR3B	E2 (394-424) E2 (437-447) E2(523-540) conformational	Cross-reactive	NA	[76]
AR3C	E2 (394-424), E2(437-447) E2 (523-540) conformational	Cross-reactive	NA	[76]
AR3D	E2 (394-424), E2 (437-447) E2 (523-540) conformational	Cross-reactive	NA	[76]
AP213	E2 (396-407) partially conformational	Gla strain	NA	[133]
H77.39	E2 (384-520) linear	Cross-reactive	NA	[131]
H35	E2-conformational	Poorly cross-reactive	NA	[134]
H48	E2-conformational	Poorly cross-reactive	NA	[134]
2/69a	E2 (436-443) linear	NA	NA	[135]
9/86a	E2 conformational	NA	NA	[130]
6/1a	E2 (464-471) linear	NA	NA	[130]
9/75	E2 (524-531) linear	NA	NA	[130]
6/53	E2 (544-551) linear	NA	NA	[130]
6/16	E2 (384-391) linear	NA	NA	[130]
1/39	E2 (432-443) linear	NA	NA	[130]
6/41a	E2 (480-493) linear	NA	NA	[130]
11/20c	E2 (436-447) linear	NA	NA	[130]
ALP98	E2 (644-651) linear	Cross-reactive	NA	[136]
ALP1	E2 (647-658) linear	NA	NA	[136]

NA: Not available.

VIRAL MECHANISMS FOR EVADING NEUTRALIZING ANTIBODIES

HCV has developed the following different mechanisms to evade neutralizing antibody responses[79]: (1) the association of HCV with low density lipoproteins[47]; (2) the association of HCV with certain glycans can play a role in shielding important neutralizing epitopes of E1 and E2 envelope glycoproteins[80]; (3) the production of interfering antibodies, which may interfere with anti-HCV neutralizing antibodies[81]; and (4) direct cell-to-cell transmission of the HCV virus[82]. HCV typically employs a combination of these mechanisms together at the same time.

Lipoproteins

It is reported that poorly infectious HCV particles linked to immunoglobulins have been separated from the higher density fractions of chronic HCV plasma samples, with highly infectious particles found in lower density

fractions, indicating the effect of lipoproteins on the infectivity of HCV[83]. The binding of HCV to lipoproteins may facilitate HCV uptake by liver cells[47,84]. This facilitated viral entry is mediated through interaction between the ApoB and SR-BI receptors, which enable HCV to escape recognition *via* different HCV neutralizing antibodies. This suggests that lipoproteins could possibly play a role in protection against antibody-mediated neutralization by masking the epitopes of the viral surface glycoproteins[85,86].

HCV genetic diversity

The high variability of the HCV genome provides the virus with an effective escape strategy from antibody neutralization[87,88]. HCV is classified into seven different genotypes, with each genotype including a number of subtypes that are roughly 25% different at the nucleotide level. The loss of the proofreading ability of HCV NS5B polymerase increases the mutation rate of the virus to one nucleotide per replication cycle[89]. This results in the evolution of HCV into many quasispecies with different though closely related nucleotide sequences even within the same patient, which may help the virus to escape from the human immune response[54,90]. HCV likely escapes the effects of the immune system because immune responses develop over weeks and HCV replicates on a timespan of days[91]. Interestingly, the sequence variation occurs mainly in the HCV E2 HVR1. HVR1 can remain with no change in its genome sequence for roughly two years given the absence of neutralizing antibodies. Given the virus's exposure to neutralizing antibodies, the HCV genomic RNA sequence undergoes several adaptive mutations[89].

Glycans

Glycans associated with HCV envelope proteins protect the virus from neutralization by various antibodies *via* shielding crucial epitopes, especially in the E2 glycoprotein. With eleven N-glycosylation sites, E2 is the most glycosylated protein of the E1-E2 heterodimer, whereas the E1 protein contains only four sites. These fifteen glycans in total were reported to play a role in the entry of HCV into host cells. Several glycans (E2N1, E2N2, E2N3, E2N4, E2N6, E2N8, E2N9, E2N10, an E2N11) on E2 were found to limit the accessibility of neutralizing epitopes on E2[92]. These nine glycosylation sites were found to be conserved across all genotypes[80], which may indicate their importance as an HCV escape mechanism[92].

Direct viral transmission from cell to cell

Direct transmission between cells helps HCV to evade both innate and adaptive immune systems. Cell-to-cell spread has been found to occur in other viral families, such as the herpes virus, retroviruses, and rhabdoviruses[93]. Direct cell-to-cell transmission is more efficient in spreading the virus in host, as it allows the virus to escape neutralizing antibodies. Furthermore, direct cell-to-cell transmission has been shown to be CD81 independent. Lastly, both CLDN1 and OCLDN cell receptors were reported to play a role in cell-to-cell transmission[94,95].

AVAILABLE AND NEW TRENDS IN ANTIBODY PRODUCTION

The high cost of production of antibodies by hybridoma or the humanization of mouse antibodies, page displays, or transgenic mice has led to the emergence of new trends to produce antibodies, such as egg-yolk-based antibody (IgY) production within transgenic plants and synthetic antibody mimics.

Egg yolk antibodies (IgY)

Providing passive immunity to chicks, IgY is passively transmitted to egg yolks to help safeguard a chick against infection until its own immune response can be developed. IgYs are functionally similar to IgG in mammals, and intensive research has been conducted on the utilization of IgY for passive immunization[96]. The transient activity of passive antibodies increases the need for large-scale production due to their frequent administration. Hen eggs are an excellent source of antibodies for passive immunization due to their being a non-invasive means of production and the large production capability of a chicken[97]. The IgY collected from egg yolks can yield eighteen times more antibodies than the serum obtained from rabbits without sacrificing the animal[98]. An average hen can lay roughly 325 eggs a year. Given that an egg can produce 60-150 mg of IgY[99], one hen can produce 20-40 g of antibodies a year, with 2%-10% of the antibodies being antigen specific[100].

Additional advantages of using IgYs to combat infections in the human body include the ease of isolating egg yolk antibodies and the absence of interaction accruing between IgYs and the Fc receptors of mammals, which can initiate an inflammatory reaction and fail to induce complement activation in mammals[101]. In addition, chicken antibodies produce a different antibody repertoire and identify different epitopes than the antibodies produced by mammals[102]. Furthermore, the large-scale industrial production of eggs makes the process of IgY production technically efficient. IgY has been used effectively against several human and veterinary viruses, such as the bovine rotavirus[103], infectious bursal disease virus[104], human influenza virus H1N1[105,106], rabies virus[107], bovine leukemia Virus[108], rabbit hemorrhagic disease virus[109], bovine respiratory syncytial virus[110], avian reovirus[111], norovirus[112], hepatitis A virus[113], and the white spot syndrome virus[114].

The use of genetic engineering technologies to produce chicken mAb (mIgYs) will enhance the utility of IgY antibodies[115]. The combination of the high specificity and homogeneity of the mAbs and the unique features of chicken antibodies provide additional features to mIgYs.

Very few studies have investigated the generation and utility of mIgYs with respect to prion immunogen[116]. The use of antibody engineering technologies facilitates the production of mIgY, while the phage display technique provides the features of a large, diverse library, as well as an efficient selection procedure[117].

Antibody expression in plants

The first trial of the production of antibodies in plants was carried out in 1989 in transgenic plants. Plant expression systems have several advantages in terms of the production of antibodies: First, these systems cost less compared to other conventional expression systems. Second, with regard to safety, they do not contain mammalian viruses or pathogens and do not produce endotoxins. Furthermore, as reported by Xu *et al*[118], the antibodies can be applied parenterally, topically, or orally. Castilho *et al*[119] and Olinger *et al*[120] report that, with antibody plant expression systems, it is possible to produce antibodies with desired glycoforms, and that glyco-engineered plants have a much higher degree of glycan homogeneity. In a recent review article by Virdi *et al*[121], they report that the production of antibodies in edible tissues would allow for oral passive immunization of the mucosal surface of the stomach. The use of technology together with the natural capacity of plant tissues to collect complex antibodies will enable in the enrichment of the antibody market. A review by Virdi *et al*[121] also showed the role of plants as an adaptable expression system for passive immunotherapy. Nianiou *et al*[122] showed the production of antibodies against HCV core gene in transgenic tobacco plants. The resultant HCV core antigen was found to be immunoreactive not only with polyclonal and mAb, but also with human sera positive for HCV-infected patients. Therefore, the authors prospected that the use of a plant-based HCV vaccine could be possible. Recently, Iranian scientists Mohammadzadeh *et al*[123] designed a highly codon-optimized HCV core protein gene for the construction of an effective plant expression system for the production of HCV core proteins with antigenic properties in an Iranian Jafarabadi-cultivar tobacco plant. The authors concluded that, through the use of a gene optimization strategy that uses vectors based on HCV and the suppression of plant-derived, gene-silencing effects, an effective expression system including the HCV core proteins of tobacco plants with antigenic immunogenic characteristics may be possible.

Synthetic antibody mimics

Recently, McEnaney *et al*[124] of the Yale University lab have crafted the first synthetic molecules (synthetic antibody mimics) that possess both the targeting abilities and functions of natural antibodies. The synthetic antibody mimics (SyAMs) attach themselves simultaneously to disease cells and immune fighting cells, performing a similar action to natural human antibodies. McEnaney *et al*[124] showed these molecules to be synthetic organic compounds that are approximately one-twentieth the size of natural antibodies. The authors report that their new SyAMs are thermally stable and can be administered orally. Furthermore, the authors report that the SyAMs have the potential to be used in treatments for cancer and other diseases, such as human immunodeficiency virus and various bacterial diseases[124]. We believe that these synthetic antibody mimics will open new areas of research and practice in the field of immunotherapy.

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