Exploring the Native Human Antibody Repertoire to Create Antiviral Therapeutics

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Abstract Native human antibodies are defined as those that arise naturally as the result of the functioning of an intact human immune system. The utility of native antibodies for the treatment of human viral diseases has been established through experience with hyperimmune human globulins. Native antibodies, as a class, differ in some respects from those obtained by recombinant library methods (phage or transgenic mouse) and possess distinct properties that may make them ideal therapeutics

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for human viral diseases. Methods for cloning native human antibodies have been beset by technical problems, yet many antibodies specific for viral antigens have been cloned. In the present review, we discuss native human antibodies and ongoing improvements in cloning methods that should facilitate the creation of novel, potent antiviral therapeutics obtained from the native human antibody repertoire.

1 Introduction: The Native Human Antibody Repertoire and Viral Disease

There is a growing awareness of the utility of and need for human antibody therapeutics for viral diseases (Keller and Stiehm 2000; Oral et al. 2002; Casadevall et al. 2004; Casadevall and Pirofski 2005). Individuals who have recovered from a viral infection, or who have received a therapeutic vaccination, contain a population of antibodies that is capable of contributing to a life-long immunity from the virus. These are defined as "native antibodies," i.e., antibodies in exactly the configurations created by a functioning, intact human immune system. Native antibodies are most commonly obtained by methods that immortalize primary B cells by hybridoma formation or Epstein-Barr virus (EBV) infection. They are distinct from human or humanized antibodies derived from recombinant DNA or transgenic mouse systems, which may not accurately replicate the complete, wild-type structure of full-length antibodies produced by the human immune system in situ. The native human antibody repertoire has tremendous potential as a source for antiviral antibody therapeutics because it contains definitive immunologic solutions to human viral diseases and is likely to be the safest overall for human clinical use. Polyclonal antibody therapeutics of unselected and disease-specific native immunoglobulins are effective in some clinical situations. These intravenous immunoglobulins (IVIG) are the starting point for exploring the potential value of the native human antibody immunome, but they do not address the vast spectrum of viral diseases for which antibodies have potential therapeutic efficacy. Over the past 25 years, ongoing efforts to improve methods for cloning native human antibodies that can capture and amplify the antiviral capabilities of IVIG have progressed, demonstrating the value of this approach and justifying further exploration. In this review we will consider the history and therapeutic potential of cloned native human antibodies specific for viral illnesses.

2 Intravenous Immunoglobulins for Human Viral Disease

IVIG is the purified population of native human IgG antibodies obtained from blood plasma. The only FDA-approved antiviral use for IVIG is to treat infection by parvovirus B19 (PV B19) in patients who are immunocompromised or have aplastic anemia (Table 1). PV B19 is a small DNA virus that in normal children

causes fifth disease, characterized by fever, malaise, and a typical bilateral cheek/facial rash (Broliden et al. 2006). In most people, parvovirus infection is self-limited. However, parvovirus can cause complications in susceptible individuals resulting from its ability to infect erythroid progenitor cells. Patients who have undergone allogeneic hematopoietic stem cell transplants or who have sickle cell anemia can have an acute aplastic crisis as a result of parvovirus infection (Broliden et al. 2006; Eid et al. 2006). Fetuses carried by pregnant women infected with the virus can develop severe anemia, complicated by an infectious myocarditis, that can induce hydrops fetalis and fetal loss (Ergaz and Ornoy 2006). For these clinical situations, IVIG is often used (Moudgil et al. 1997; Geetha et al. 2000; Broliden et al. 2006; Eid et al. 2006). Human antibodies have been cloned that bind the major or minor capsid proteins and can neutralize parvovirus in vitro (Arakelov et al. 1993; Gigler et al. 1999). These may be candidates for cloned parvovirus antibody therapeutics.

Hepatitis A is a picornavirus, containing a positive-strand RNA genome, which causes an acute, self-limited hepatitis (Fiore et al. 2006). Prior to the creation of the recombinant hepatitis A vaccine, IVIG was routinely given for hepatitis A prophylaxis, although it was not FDA-approved for this purpose (CDC 1985; Fiore et al. 2006; Table 1). IVIG is still indicated for suspected, nonimmune contacts and for people who are intolerant of the vaccine. Native antibodies have been cloned that are specific for the capsid protein (Cerino et al. 1993) or the core antigen (Siemoneit et al. 1994), and one antibody has been cloned that is capable of neutralizing hepatitis A in vitro (Lewis et al. 1993).

The potential utility of IVIG for treatment of viruses other than PV B19 and hepatitis A is broad and reflects the collective antibody immunome of the population

Table 1 Common indications for the use of intravenous immunoglobulins (IVIG) or disease-specific, hyperimmune immunoglobulins (Hyper-IG). References are cited in the text

Virus	Globulins	Patients	Purpose
Parvovirus B19	IVIG	HSCT patients	Treatment
		Sickle cell disease patients	Treatment
Hepatitis A	IVIG	Anyone at risk	Prophylaxis
Hepatitis B	Hyper-IG	Possibly exposed to HBV	PEP
		Neonates	Inhibit vertical transmission
		HBV-infected liver transplant	Prophylaxis
CMV	Hyper-IG	Solid organ transplant	Treatment
VZV	Hyper-IG	Immunocompromised	Treatment of severe cases
		Neonates	Inhibit vertical transmission
VV	Hyper-IG	Immunocompromised	Treatment
Rabies	Hyper-IG	Anyone at risk	PEP
RSV	Hyper-IG	Premature infants/BPD	Prophylaxis
		HSCT patients	Treatment

BPD, bronchopulmonary dysplasia; CMV, cytomegalovirus; HBV, hepatitis B virus; HSCT, patients who have undergone allogeneic hematopoietic stem cell transplantation; PEP, post-exposure prophylaxis; VV, vaccinia virus; VZV, varicella zoster virus

from which it is derived. A study of five different IVIG preparations revealed antiviral antibodies specific for types 1, 2, 6, and 7 herpesviruses (HSV), varicella zoster (VZV), EBV, measles, mumps, rubella, and parvovirus B19 (Krause et al. 2002). Differences in the IVIGs were also noted, in that two had high levels of antibodies specific for adenovirus and two had high levels of St. Louis encephalitis virus antibodies. A panel of eight IVIG preparations was recently examined for the presence of vaccinia virus (VV) neutralizing ability (Goldsmith et al. 2004). All were found to contain significant in vitro and in vivo VV neutralizing activity, at 3%–9% of the measured titer of standard VV hyperimmune globulins (VIG), even though widespread VV vaccination has not been practiced for nearly the past 30 years. IVIG may also have clinical activity against West Nile virus (WNV). A WNV patient in Israel recovered from the infection after treatment with IVIG. It is notable that WNV is endemic in Israel and the Israeli IVIG had a high titer of WNV antibodies. In contrast, IVIG from the United States was not found to contain WNV antibodies. IVIG has also been considered to be of potential benefit for cytomegalovirus (CMV) infection in renal and bone marrow transplants (Sechet et al. 2002; Sokos et al. 2002).

3 Hyperimmune Globulins and Native Human Antibodies for Viral Diseases

The complications of some viral infections are preferably treated with hyperimmune globulins, which are polyvalent IVIGs obtained from subjects with high-titer antibody responses to specific antigens (Table 1). Some of the viruses treated with this category of therapeutics are hepatitis B (HBV), CMV, VZV, VV, rabies, and respiratory syncytial virus (RSV). The first three of these viruses share the capability of reactivation in patients who become immunocompromised. In these settings, hyperimmune globulins are used to ameliorate the immunodeficiency and bring the reactivated viruses under control. VV causes an acute infection that can have severe manifestations in immunocompromised hosts and can be mitigated by VV-specific hyperimmune globulins. Rabies induces an acute infection that is invariably fatal, unless treated, even in immunocompetent individuals. Rabies immune globulins (RIG) provide initial control of the virus while a concomitantly administered vaccine induces permanent immunity. RSV can cause fatal bronchiolitis in premature infants and recipients of allogeneic hematopoietic stem cell transplants. In these patient populations, RSV immunoglobulins (RSV-IG) are effective for RSV prophylaxis and treatment. The different roles for hyperimmune globulins are empirically defined and reflect the unique clinical features of each virus and the infected hosts. For most of these viruses, native human antibodies have been cloned that may possess some of the functions provided by the hyperimmune globulins. Future optimized hyperimmune globulins would consist of these or similar antibodies in completely defined monoclonal or oligoclonal antiviral therapies.

3.1 Hepatitis B Virus

HBV is a partially double-stranded DNA virus that is transmitted by direct contact with infected bodily fluids (Hollinger and Liang 2001). In normal individuals, hepatitis B will generally induce a self-limited hepatitis, but it has the capacity to establish a chronic active state that can eventually lead to cirrhosis or hepatocellular carcinoma. In the United States, hepatitis B immune globulin (HBIG) is prepared from a small number of donors hyperimmunized with the HBsAg vaccine (Terrault and Vyas 2003). The main three categories of use for HBIG are (1) post-exposure prophylaxis for nonimmune contacts, (2) inhibition of vertical transmission of the virus at birth, and (3) prevention of relapse in HBV-positive patients following orthotopic liver transplantation. Following a suspected infected needle-stick or fluid exposure, HBIG is recommended to be administered in combination with the HBsAg vaccine (CDC 1984). Maternal-fetal transmission can be inhibited by administration of HBIG and the HBsAg vaccination immediately after birth to infants of mothers positive for circulating HBe antigen and HBV DNA (Lo et al. 1985; Ip et al. 1989; Kabir et al. 2006). Further improvements in outcomes may also be achieved by passive immunization of HBe-positive mothers with HBIG prior to delivery (Xu et al. 2006; Xiao et al. 2007). The successful use of orthotopic liver transplantation to treat end-stage liver disease caused by HBV depends on the prevention of reactivation of the virus in the immunocompromised, post-transplant patient (Gish and McCashland 2006). HBIG can collaborate with nucleoside antiviral agents to limit reactivation in these patients, but there remains a risk of reactivation of disease after the discontinuation of prophylactic therapy. In this setting, the cost of long-term HBIG administration may potentially be reduced through the use of low-dose combination therapy regimens (Di Paolo et al. 2004; Ferretti et al. 2004).

Many native human IgG antibodies specific for HBV have been cloned using hybridoma and EBV-immortalization methods (Stricker et al. 1985; Colucci et al. 1986; Desgranges et al. 1987; Ichimori et al. 1987; Tiebout et al. 1987; Andris et al. 1992; Ehrlich et al. 1992; Sa'adu et al. 1992; Heijtink et al. 1995). Animal data are difficult to obtain with HBV infection, but a combination of two human antibodies administered to a chimpanzee chronically infected with HBV was able to transiently (<7 days) reduce the levels of circulating virus (Heijtink et al. 1999). An important concern regarding the efficacy of cloned antibodies for HBV is the apparent ability of the virus to escape neutralization by polyclonal HBIG. This phenomenon has been observed in liver transplant patients who had recurrent HBV infection after liver transplantation despite HBIG therapy. In three studies, the existence of mutations in antigenic regions of HBsAg correlated with resistant or recurrent HBV infection (Carman et al. 1996; Ghany et al. 1998; Terrault et al. 1998). Furthermore, the length of time of therapy correlated with the likelihood of finding mutated HBV strains (Ghany et al. 1998; Terrault et al. 1998). The potential failure of polyclonal antibodies may suggest that the virus would be particularly adept at escaping the effects of a monoclonal or oligoclonal antibody therapeutic. A pair of antibodies with significant HBV binding had considerably less affinity for

a variant HBV strain that had arisen in a patient following a year of HBIG therapy (Heijtink et al. 1995). Nonetheless, it may be possible to create an oligoclonal HBIG equivalent or superior to polyclonal HBIG by identifying two or three non-cross-resistant antibodies directed at relatively stable portions of HBsAg.

3.2 Cytomegalovirus

CMV is a double-stranded DNA virus that, in normal individuals, induces a febrile illness that resembles mononucleosis from EBV, with chills, fatigue, headache, and malaise (Gandhi and Khanna 2004). In immunocompromised patients, such as those who have undergone bone marrow or other organ transplantation or who have advanced human immunodeficiency virus (HIV) disease, CMV can cause considerable morbidity and mortality. CMV may be reactivated in a previously infected person who becomes immunosuppressed. Alternatively, a de novo CMV infection may be transmitted to a CMV-negative recipient of an organ from a CMV-positive donor. Many organs can be affected by CMV infection, including the retina, lung, liver, esophagus, or colon. CMV can also cause complications to a fetus infected in utero, including hearing loss, visual loss, and neurological complications (Fowler et al. 1992). CMV hyperimmune globulins (CMVIG) first demonstrated efficacy in the treatment of disease associated with kidney transplants (Snydman et al. 1987). Since then, CMVIG has been approved by the FDA for treatment of CMV reactivation in patients with transplants of the kidney, heart, lung, liver, and pancreas (Sawyer 2000). Evidence does not clearly support the use of IVIG or CMVIG in allogeneic bone marrow transplant patients (Zikos et al. 1998; Sokos et al. 2002). The utility of CMVIG in organ transplant settings has been lessened by the availability of potent small molecule anti-CMV drugs, such as ganciclovir, valganciclovir, foscarnet, and cidofovir, even though these drugs have significant toxicities (Biron 2006). It is possible that CMVIG may synergize with small molecule anti-CMV drugs in some clinical situations (Kocher et al. 2003; Varga et al. 2005; Ruttmann et al. 2006).

Many native human monoclonal antibodies specific for CMV have been described (Emanuel et al. 1984; Redmond et al. 1986; Foung et al. 1989; Bron et al. 1990; Kitamura et al. 1990; Drobyski et al. 1991; Gustafsson et al. 1991; Ohizumi et al. 1992; Ohlin et al. 1993; Rioux et al. 1994). Some of these were found to be capable of neutralizing CMV in vitro (Redmond et al. 1986; Foung et al. 1989; Ohizumi et al. 1992; Ohlin et al. 1993). The native human CMV antibody, MSL-109, has been tested for clinical efficacy (Drobyski et al. 1991). In a randomized controlled trial of allogeneic hematopoietic stem cell transplant patients, no benefit from the antibody was seen in terms of the time to development of CMV viremia or pp65 antigenemia (Boeckh et al. 2001). Studies of the MSL-109 antibody in AIDS patients with newly diagnosed or recurrent CMV retinitis did not show a reduction in the progression of CMV disease (CDC 1997a; Borucki et al. 2004). The explanation for these disappointing results is unclear.

The MSL-109 antibody is specific for the H glycoprotein (gp86). It is possible that an antibody specific for the B glycoprotein complex (gp58/116), a major target of CMV neutralizing antibodies, may be useful alone or in combination with an anti-H antibody (Ohlin et al. 1993). Nonetheless, the most likely explanation may be that T cell function is essential for CMV control in vivo and that neutralizing antibodies are minimally active in the absence of robust T cell activity (Boeckh et al. 2003).

3.3 Varicella Zoster Virus

VZV is a highly transmissible, double-stranded DNA poxvirus that induces a febrile illness (chickenpox), which is characterized in children by fever, malaise, and a pruritic, vesicular rash (CDC 1996). VZV can also reactivate in adulthood as a series of painful vesicular lesions in the distribution of a cutaneous dermatome. Infection of pregnant women during the first and second trimesters may induce the congenital varicella syndrome, which can result in significant fetal deformities, and VZV infection transmitted to newborns can be fatal (Tan and Koren 2006). Accordingly, VZV immunoglobulins (VZIG) are indicated for immunocompromised patients, pregnant women, and neonates at risk for VZV infection (CDC 2006; Tan and Koren 2006). Native human antibodies capable of neutralizing VZV in vitro have been cloned (Foung et al. 1985; Sugano et al. 1987, 1991).

3.4 Vaccinia Virus

One of the established uses of human hyperimmune globulins is the treatment of complications of vaccinia virus (Lane et al. 1969; Henderson et al. 1999). Vaccinia virus is a poxvirus that has been adapted for use as a human vaccine for the prevention of smallpox. Although generally safe for immunocompetent persons, disseminated and occasionally fatal infections can occur among patients with underlying immunodeficiencies, such as those with HIV infection, eczema, or atopic dermatitis (Henderson et al. 1999). Generalized vaccinia is a syndrome in which VV proliferation is systemically spread through the bloodstream (Redfield et al. 1987). Progressive VV infection is characterized by unrestrained proliferation of virus in the skin. Eczema vaccinatum is the excessive proliferation of VV in the skin lesions of eczema patients. For these conditions, VIG is indicated and can often lead to a complete resolution of symptoms (Henderson et al. 1999). VIG is also useful in immunocompetent individuals who have a complicated infection, such as may result from accidental infection of the periorbital region (Lewis et al. 2006).

Creation of cloned neutralizing antibody therapeutics for VV may be challenged by its complex life cycle. The VV virion exists in two forms that differ in their ability to be neutralized by antibodies, the intracellular mature virion (IMV) and the extracellular enveloped virion (EEV), with the IMV more susceptible to neutralization

than the EEV (Law and Smith 2001; Smith et al. 2002). Optimal protection against lethal VV in murine and rhesus macaque models by a DNA vaccine required a combination of four genes directed at both the IMV and EEV (Hooper et al. 2003). A cloned native murine antibody specific for the A27L antigen (a neutralization target of the IMV) was able to protect mice prophylactically and therapeutically from a lethal VV challenge (Ramirez et al. 2002). However, no comparable native or nonnative human antibodies have been described. It will be important, however, to determine how many cloned human antibodies will be required to improve the symptoms of VV infection in immunocompromised patients.

3.5 Rabies Virus

Rabies is a virus with a single-stranded RNA genome that causes an acute and universally fatal encephalitis. The efficacy of RIG for the post-exposure prophylaxis of rabies has been reviewed elsewhere in this volume (see the chapter by T. Nagarajan et al.). Briefly, human rabies immunoglobulins (HRIG) are used in combination with rabies vaccination for a known or suspected rabies exposure, administered intravenously as well as directly into the suspected exposure site (see the chapter by T. Nagarajan et al., this volume). Native human antibodies have been cloned that are capable of neutralizing the virus in vitro and in vivo; most of these are reactive with the rabies glycoprotein (Dietzschold et al. 1990; Gebauer and Lindl 1990; Lafon et al. 1990; Ueki et al. 1990; Enssle et al. 1991; Dorfman et al. 1994; T. Nagarajan et al., this volume). A combination of two human antibodies that bind noncross-resistant epitopes on the glycoprotein has undergone preclinical in vivo testing. One of the antibodies was a native antibody and the other was cloned using the phage display method (Champion et al. 2000; Bakker et al. 2005). The antibody combination demonstrated efficacy comparable to HRIG and did not interfere with the potency of a simultaneously administered rabies vaccine (de Kruif et al. 2006).

3.6 Respiratory Syncytial Virus

RSV is a single-stranded, negative-strand virus that usually causes an upper respiratory infection (Welliver 2003). In some patient populations, RSV infection can develop into a bronchiolitis, an inflammation of the bronchioles, the smallest air passages of the lung. It is an important cause of mortality in young children and the elderly, and no vaccine for the disease currently exists (Shay et al. 2001; Thompson et al. 2003; Falsey et al. 2005). RSV exists in two main subtypes, A and B, but infection with one subtype does not even provide lifelong protection from reinfection by the same subtype (Welliver 2003). Premature infants and those affected by bronchopulmonary dysplasia (BPD) are at increased risk for hospitalization and death from RSV bronchiolitis (Aujard and Fauroux 2002). The prevalence of RSV infection in this population can be reduced by prophylactic

treatment with RSV-IG (CDC 1997b). In contrast, RSV-IG did not show any efficacy in the treatment of infants already admitted to the hospital with the disease (Rodriguez et al. 1997). RSV may also cause a fatal bronchiolitis in patients undergoing allogeneic stem cell transplantation, and off-label administration of RSV-IG with the antiviral drug ribavirin may reduce mortality (DeVincenzo et al. 2000; Ghosh et al. 2000; Small et al. 2002).

No native human antibodies that neutralize RSV have been cloned. However, a humanized murine monoclonal antibody, palivizumab, is a potent substitute for RSV and the first demonstration of the utility of a monoclonal antibody as an antiviral therapeutic (Young 2002; see the chapter by H. Wu et al., this volume). Palivizumab binds an epitope on the F glycoprotein, a viral surface protein that is a major target for neutralizing antibodies and is highly conserved between type A and B viruses. In a series of high-risk infants with prematurity and/or BPD, a course of monthly prophylactic doses of palivizumab reduced the overall rate of serious infections and hospitalizations by 55% (CDC 1998). A role for palivizumab in the treatment of RSV infection in the elderly or in allogeneic hematopoietic stem cell patients has not yet been established.

3.7 Cloning Antibody Therapeutics for Viral Disease

The potency of IVIG, hyperimmune IGs, and the monoclonal antibody palivizumab demonstrate in principle that human antibody therapeutics are likely to be effective for the treatment of viral diseases. A vast, unmet medical need exists for treatments for the majority of viral diseases that occur worldwide. The development of antiviral antibody therapeutics will be challenged by the diversity of virus types, patient populations and the roles antibodies play in the neutralization of specific viruses, the ability of viruses to mutate antigenic domains, and an incomplete understanding of the specific features that endow an antibody with neutralizing ability. To counter these uncertainties it will be important to explore as diverse an antibody repertoire as possible, which can best be achieved by using a variety of different, complementary methods for human antibody cloning. The efficacy of IVIG and hyperimmune IGs suggests that an ideal starting point to clone a human antibody capable of potently neutralizing a viral pathogen may be with B cells from subjects who have developed a definitive antiviral body response, either by infection or vaccination. Native antibody libraries created from these affinity-matured B cells would be expected to contain individual antibodies that possess virus-neutralizing abilities and would be suitable for use as monoclonal or oligoclonal antibody therapeutics. The successes of native human antibody cloning methods in obtaining native human antiviral antibodies, and the potency of these antibodies, establish a rationale for further exploration of these methods. It is evident that the effectiveness of this approach will depend on the ability to create libraries that come as close as possible to comprehensively incorporating the entire diversity of the human antibody response to viral pathogens.

4 Features of Different Cloned Human Antibody Repertoires

The prevalent methods of cloning human antibodies from immune human repertoires differ in bias and in the degree to which they sample antibodies in their native configurations (e.g., with the original heavy chain:light chain pairing). B cell immortalization methods, which use hybridoma generation or EBV infection to enable primary human B cells to proliferate in vitro, theoretically take an unbiased sample of the repertoire of B cells and express each antibody with native heavy chain:light chain pairings. In addition to the antibodies described in the preceding section, these methods have been used to clone native human antibodies specific for measles, HIV, severe acute respiratory syndrome (SARS), EBV and hepatitis C. These methods have historically been challenged by poor antibody yields and unstable antibody secretion. Nonetheless, they have been the focus of ongoing optimization efforts that should improve their ability to comprehensively access the native human antibody immunome.

Recombinant DNA methods offer a well-established method for cloning human antibody repertoires. In these methods, heavy chain and light chain variable domains are amplified from B cell populations using RT-PCR, fused, and expressed as single-chain antigen-binding domains (scFv) on the surface of filamentous phages (Barbas 1993; Winter et al. 1994). Screening for specific antibodies is performed by panning for virus that binds to a plate or other solid support coated with antigen (Bradbury and Marks 2004). A related technology is yeast display, in which the scFv molecules are expressed on the surface of *Saccharomyces cerevisiae* (Boder and Wittrup 1997). Yeast display allows greater diversification of expressed antibody sequences by mutagenesis and has the advantage that yeast cells expressing human antibody can be directly screened by flow cytometry with fluorescent antigen, enabling a rapid assessment of binding kinetics. Libraries of scFv antibodies have also been efficiently expressed on the surface of *Escherichia coli* (Daugherty et al. 1999).

It is clear that recombinant DNA libraries obtained from immune individuals differ from antibodies obtained from nonimmune individuals (Amersdorfer et al. 2002). However, the process of creating these libraries can introduce bias at different steps in the process that may hinder their ability to capture the entire native antibody repertoire. The first step is an RT-PCR amplification with consensus DNA primers, which may not equally amplify each immunoglobulin gene sequence. The second is at the level of expression in phage, because *E. coli* does not express all eukaryotic peptides with the same efficiency, and human variable domain gene sequences can differ significantly from one another in their length and amino acid composition (Pavoni et al. 2006). A combination of these effects could potentially reduce the prevalence of specific antibodies in the antibody libraries or eliminate them entirely.

Evidence that this occurs comes from DNA sequence analysis of complementarity-determining (CDR) regions of heavy chain variable domains (V_H) cloned by phage display. The third CDR region (CDR3) of the V_H is the most important contributor to the antigen-binding specificity of an antibody (Xu and Davis 2000). CDR3 regions incorporated into phage display libraries tend to be short (less than

15 amino acids), whereas CDR3 regions in native human antibodies vary widely in length, with many over 20 amino acids in length (Griffiths et al. 1994; Brezinschek et al. 1995; Tian et al. 2007). Shorter CDR3 regions correlate overall with greater levels of somatic hypermutation, but longer CDR3 regions may be better capable of viral neutralization (Saphire et al. 2001; Hangartner et al. 2006; Tian et al. 2007). It is possible that shorter CDR3 regions are selected against at the level of PCR amplification or expression in *E. coli.*

Two studies have directly examined the types of immune libraries that arise from phage display and hybridoma methods. In one comparative study, antibodies cloned from mice immunized with human interleukin-5 protein using phage display and hybridoma methods were compared (Ames et al. 1995). Each method produced a structurally distinct group of antibodies, and only the antibodies cloned by the hybridoma method were able to block binding of the cytokine with its receptor. Ohlin and Borrebaeck (1996) analyzed a dataset of cloned antibody sequences specific for infectious disease antigens, the majority of which were viral, and were cloned by either the phage display or hybridoma method (Ohlin and Borrebaeck 1996). They noted substantial differences in the heavy chain and light chain gene family utilization between antibodies derived from the two different sources. They also noted a dramatic limitation of the diversity of the light chain gene repertoire. This observation may have been due to the phenomenon of light chain promiscuity, i.e., the ability of heavy chains to productively associate with a variety of light chains (Kang et al. 1991).

The ability of a phage library to recreate native heavy chain:light chain combinations was recently assessed by comparing a phage display antibody library that maintained native pairings with one made from the same cDNA that did not (Meijer et al. 2006). In the random library, the assortment of heavy chain and light chain sequences had apparently lost a majority of the original heavy chain:light chain pairings. Consistent with the principle of light chain promiscuity, the diversity of the random library was less than the nonrandom library due to an over-representation of VH chains capable of associating with many different light chain sequences. The functional importance of the antibody repertoire shift in the random library was revealed by the overall lower affinity of antibodies specific for tetanus toxoid (TT) antibodies cloned from the two libraries.

Taken together, these experiments illustrate the concept that intrinsic biases in phage display libraries may prevent some important native antibody structures from being incorporated into them. B cell immortalization methods of human antibody cloning are therefore complementary to recombinant DNA methods and thus merit further study and optimization.

5 Hybridoma Methods to Clone Native Human Antibodies

As a starting point in native human antibody cloning methods, the source of virus-immune B cells is an immune individual who has generated an antibody response that is effective in collaborating with the human immune system to cure the viral

infection. Thus, there can be a presumption that antibodies with the requisite biological functions exist within the volunteer B cell donor. Donors can be subjects who have either (1) received vaccines specific for the virus, (2) survived an infection by the relevant virus, or (3) have succumbed to the viral infection but have made spleen, lymph nodes, or peripheral blood mononuclear cells (PBMCs) available *post mortem*. The use of B cells from a variety of genetically unrelated individuals can increase the diversity of the native antibody libraries to be screened.

There are many approaches to cloning human antibodies in their native configurations. For the most part, these involve methods of converting primary human B cells into a form that is viable in vitro through EBV immortalization, hybridoma formation, or a combination of these protocols. In EBV immortalization, purified B cells are infected with EBV-containing supernatant from the B95-8 marmoset cell line (Brown and Miller 1982). These methods are effective, yet they can be compromised by the low levels of antibody that are typically expressed by EBV-transformed cells (lymphoblastoid cells, LCLs) (Stein and Sigal 1983). In hybridoma methods, primary human B cells are fused to an immortal fusion partner cell line, which is adapted to in vitro culture and capable of producing high levels of antibody from immunoglobulin genes provided by the primary B cell. The primary impediments to hybridoma approaches have been low hybrid cell yields and the loss of antibody expression, which correlates with the loss of human chromosomes from the hybrid cells. Combination approaches have been taken that can overcome some of these defects by immortalizing and expanding the antigen-specific B cell population first with EBV infection, and then fusing the immortalized cells to a murine or murine/human fusion partner cell line.

5.1 Improvements in Fusion Partner Cell Lines

Most of the technology development in this area has attempted to address the problem of hybridoma instability by improving the fusion partner cell line. It had originally been considered that human cell lines would be optimal as fusion partners for primary human B cells because hybrid cells formed between murine cells and human cells were known to segregate human chromosomes (Ephrussi and Weiss 1969). The first reported human antibody cloning by a hybridoma method was an IgM antibody specific for measles virus (Croce et al. 1980). For this purpose, Croce et al. used a human myeloma cell line as a fusion partner cell and PBMCs from a patient with subacute sclerosing panencephalitis, the clinical syndrome resulting from measles virus infection of the central nervous system. Shortly thereafter appeared the first report of use of an EBV-immortalized B cell line as a fusion partner to clone antibodies specific for TT (Chiorazzi et al. 1982). An EBV-immortalized human B cell expressing an antibody to CMV was fused to a human myeloma cell line to give a hybrid with improved antibody expression (Emanuel et al. 1984). Enthusiasm for human cell lines was tempered, however, due to problems with the limited number of immortalized myeloma and other B cell lines that were available (Kozbor et al. 1986). Most of the cell

lines had low fusion rates and produced slow-growing hybridomas, and many already expressed human antibody genes. Chromosomal instability was also observed to be a considerable problem (Olsson et al. 1983).

Experiments using murine myeloma cell lines as fusion partners for human B cells demonstrated a poor efficiency that likely resulted from the strong tendency of murine/human hybrid cells to rapidly segregate human chromosomes (Ephrussi and Weiss 1969; Schlom et al. 1980; Kozbor et al. 1982; Koropatnick et al. 1988). To compensate for the defects intrinsic to human and murine partner cell lines, a variety of heteromyeloma (murine and human) cell lines have been created. The general approach taken was to fuse murine myeloma cell lines with human cells, either normal PBMCs (Foung et al. 1984; Ichimori et al. 1985; Grunow et al. 1988) or malignant cells (Carroll et al. 1986; Posner et al. 1987; Faller et al. 1990; Shirahata et al. 1998).

Where examined, these fusions have generally resulted in hybrid cells with chimeric murine/human genomes that appear to be improved fusion partners for creating hybridoma cells that stably secrete human antibodies. For instance, the CB-F7 and the SPAM-8 heteromyelomas contained no distinct human chromosomes, but did contain human DNA detectable by hybridization analysis, probably in the form of murine/human chimeric chromosomes (Grunow et al. 1988; Gustafsson et al. 1991). The heteromyeloma cell lines K6H6/B5, HAB-1, HM-5, and SPC-H20 all possessed independent, metacentric chromosomes, consistent with a human origin (Foung et al. 1984; Ichimori et al. 1985; Carroll et al. 1986; Faller et al. 1990). When directly compared to the parental murine myeloma cell lines, the heterohybridoma fusion partner cell lines tended to have an improved ability to give rise to hybrid cells that stably expressed human antibodies (Foung et al. 1984; Carroll et al. 1986; Grunow et al. 1988; Faller et al. 1990). As many of the hybrid cells derived from these fusion partner cells contained substantial numbers of human chromosomes, it is likely the heteromyeloma cell lines were better able to produce hybrid cells with a reduced tendency to segregate human chromosomes (Foung et al. 1984; Carroll et al. 1986; Grunow et al. 1988; Faller et al. 1990). Using heteromyeloma fusion partner cell lines, a wide variety of native human antibodies have been cloned that were specific for important viral pathogens. These included the human T cell lymphotropic virus (HTLV-1), CMV, HBV, hepatitis C virus (HCV), HIV, and VZV (Foung et al. 1984; Carroll et al. 1986; Grunow et al. 1988; Bron et al. 1990; Faller et al. 1990; Gustafsson et al. 1991; Hadlock et al. 1997, 2000).

5.2 Methods of Preparing Human B Cells for Fusion

Along with the improvements in the fusion partner cell lines, the parameters affecting the rate of productive hybrid cell formation have been systematically analyzed. The best sources of primary human B cells are the splenic mononuclear cells, tonsils, or peripheral blood mononuclear cells from infants (Olsson et al. 1983; Grunow et al. 1988; Jessup et al. 2000; Karpas et al. 2001). The time of

harvest of B cells following a vaccination is also important, with the best outcomes with TT antibodies seen with cells obtained 5–7 days following the vaccination, which corresponds to the period of time when the maximum quantity of TT-specific memory B cells is circulating in the blood (Butler et al. 1983; Lanzavecchia et al. 2006). Treatment of the primary B cells with a proliferative stimulus prior to fusion is also essential, either with pokeweed mitogen (PWM) or EBV (Butler et al. 1983; Larrick et al. 1983; Olsson et al. 1983; Cole et al. 1984; Emanuel et al. 1984). PWM is superior to phytohemagglutinin and is optimally used for 5–7 days (Olsson et al. 1983; Arinbjarnarson and Valdimarsson 2002). Costimulation of mitogen-treated cells with antigen can increase the yield of antigen-specific antibodies (Butler et al. 1983; Sugano et al. 1987). During the cell fusion, the ratio of B cells to immortal fusion partner cells is an important variable (Butler et al. 1983; Perkins et al. 1991).

Two groups of investigators have noted improvements in fusion efficiencies when the PBMCs are expanded prior to cell fusion using the CD40 system, an in vitro cell culture method that uses antibodies specific for CD40 and interleukin (IL)-4 to stimulate B cell proliferation and survival in vitro prior to cell fusion (Banchereau and Rousset 1991; Darveau et al. 1993; Thompson et al. 1994). Some of the benefit from expansion of the B cells in the CD40 system or by EBV-immortalization may derive from removing cytotoxic cells from the fusion that may threaten the viability of nascent heterohybridoma cells, which presumably express a variety of murine protein antigens, in the context of human MHC, that may be recognized as foreign by the human cytotoxic cell population. Consistent with this hypothesis, Borrebaeck and his colleagues demonstrated a dramatic improvement in the yields of murine/human and human/human cell fusions when they treated the input PBMCs with l-leucine methyl ester (Leu-OMe), which is toxic to lysosome-rich cytolytic cells, including natural killer (NK) cells and some T cells (Borrebaeck et al. 1987; Borrebaeck et al. 1988). A similar potential effect on cytotoxic, unfused cells was observed by Kalantarov et al. with the murine/human fusion partner cell line MFP-2S, which carried the *neo* drug-resistance marker. Inclusion of G418 in the cell culture medium post-fusion substantially reduced the variability of yields of antibody-secreting hybrid cells (Kalantarov et al. 2002).

In principle, it may be helpful to enrich cell populations for expression of specific antibodies prior to cell fusion. In a report approximately 20 years ago, Casali et al. selected B cells expressing antibodies specific for TT prior to EBV immortalization (Casali et al. 1986). More recently, in comparison to results obtained with unselected PBMCs, fusions performed with CD19-selected B cells had increased hybridoma yields (Schmidt et al. 2001).

5.3 Electrofusion and Hybrid Cell Culture

As an alternative to traditional chemical methods of inducing cell fusion with polyethylene glycol (PEG), electrofusion can offer dramatically improved rates of cell fusion (Pratt et al. 1987; Foung et al. 1990; Perkins et al. 1991). In electrofusion,

the cells to be fused are aligned in a hypoosmolar buffer solution using an alternating current. Fusion is then induced by one or more bursts of direct current. Electrofusion has been used with a variety of fusion partner cell lines, including lymphoblastoid, heteromyeloma and murine myeloma cell lines (Pratt et al. 1987; Foung et al. 1990; Yoshinari et al. 1996). Three studies have directly compared the efficiency of electrofusion and polyethylene glycol (PEG) fusion, estimating an apparent superiority of electrofusion of 4- to 100-fold, with a maximal calculated fusion rate of approximately 1 cell per 1,000 input human B cells (Perkins et al. 1991; Krenn et al. 1995; Panova and Gustafsson 1995). To improve the viability of hybrid cells following fusion, a delay of 24 h prior to the initiation of HAT selection (hypoxanthine, aminopterin, thymidine) and the use of cell feeder layers to support hybridoma growth have also been found to be helpful (Cote et al. 1983; Perkins et al. 1991; Hoffmann et al. 1996; Shirahata et al. 1998).

6 Recent Advances in Native Human Antibody Cloning

Improvements continue to be made in the fusion partner cell lines. The Karpas 707H cell line is a near-tetraploid human myeloma cell line that has been specifically selected for improved growth rates in vitro and resistance to PEG, which is required for cell fusion but which was toxic to the original myeloma cell line (Karpas et al. 2001). Karpas 707H effectively fuses with tonsillar B cells and lymphoblastoid cells and is notable for the creation of hybridomas that secrete up to 210 µg antibody/ml culture medium. An analysis of the antibodies cloned from thymocytes fused to the Karpas 707H cell line revealed a spectrum of antibodies representing different stages in the B cell differentiation process (Vaisbourd et al. 2001).

MFP-2B is a novel heterohybridoma cell line that is actually the progeny of two cell fusions. The first was between a murine myeloma cell line and a human myeloma cell line. The second was between one of the resultant heterohybridomas and primary human lymphocytes obtained from a lymph node (Kalantarov et al. 2002). The MFP-2B has been additionally modified to express a *neo* resistance gene, enabling negative selection against cytotoxic cells following the cell fusion (Kalantarov et al. 2002). This cell line is notable for its fusion and cloning efficiency. A karyotype demonstrates no intact human chromosomes, but 40% of the chromosomes are partial human chromosomes or chimeric murine/human chromosomes. The MFP-2B cell line has also been used to clone antibodies specific for breast cancer antigens (Kirman et al. 2002).

An important alternative to hybridoma methods is a recently improved EBV-immortalization method, in which human primary $CD19^{+}IgG^{+}B$ cells are stimulated with a CpG oligonucleotide prior to EBV exposure (Hartmann and Krieg 2000; Traggiai et al. 2004). The polyclonal B cell proliferation increases the rate of EBV immortalization from 1%–2% to 30%–100%. In addition, the efficiency of cloning the transformed cells was improved by including CpG oligonucleotides in the culture medium and using an irradiated mononuclear cell layer. Others have

noted that lymphoblastoid cells tend to have unstable IgG expression, but the immortalized cells were found to produce 3–20 μg antibody/ml supernatant and were stable enough to enable in vitro and in vivo functional experiments (Stein and Sigal 1983; Traggiai et al. 2004). This method enabled the cloning of a panel of IgG antibodies specific for either the nucleoprotein or the spike protein of the SARS virus, some of which were able to significantly reduce proliferation of the virus in a murine disease model (Traggiai et al. 2004).

Improvements have also been made in phage display methods that may mitigate some of the factors that hinder the incorporation of native human antibody genes into typical phage display libraries. As described above, Meijer et al. employed a novel approach of in-cell, single-cell PCR with consensus oligonucleotides that produce an individual, correctly paired scFvs from each cell (Meijer et al. 2006). These scFvs were then used to create a phage display library for screening. Analysis of the paired sequences produced by this method demonstrated consistent pairing of the same heavy and light chains, indicating preservation of the native paired antibody conformations.

7 The Use of Ectopic Gene Expression to Improve Hybridoma Stability

Little is understood about the causes of the intrinsic instability of hybridomas formed with primary human B cells or of the segregation of human chromosomes by murine/human hybrid cells (Ephrussi and Weiss 1969; Cieplinski et al. 1983; Harris et al. 1990). To begin to address these questions empirically, we and others have considered that empiric modification of fusion partner cells by ectopic gene expression may provide insight into the nature of hybridoma cells while potentially improving their utility.

The first experiments along these lines were based on the observation that addition of IL-6 to the culture medium of murine/murine cell fusions could increase the proportion of hybrid cells expressing murine antibody (Bazin and Lemieux 1989). Interleukin-6 is essential for myeloma cell growth, possessing proliferative and antiapoptotic functions, in addition to the ability to directly stimulate antibody gene expression (Hirano 1998). Addition of IL-6 to hybridoma culture medium improved the cloning efficiency and antibody secretion of established hybridomas (Zhu et al. 1993). SP2/0 cells ectopically expressing high level mIL-6 (SP2/mIL-6) were found to give improved yields of hybridomas secreting both antigen-specific and nonspecific antibodies, compared with untransfected, parental SP2/0 cells (Harris et al. 1992).

A similar experiment was performed with the goal of improving the stability of murine/human cell fusions (Zhu et al. 1999). Interleukin-11 (IL-11), which shares many functions with IL-6, was ectopically expressed in a murine fusion partner cell line. Expression of IL-11 improved the yields of hybridomas following selection, and this effect was noted with both mitogen-stimulated and EBV-transformed

B cells. IL-11 expression also increased the quantity of antibody produced by hybrids derived from stable LCLs. However, no data were given on the long-term stability of the hybridomas. It is likely that they were still prone to segregation of human chromosomes and the associated loss of antibody expression. We performed similar experiments, comparing the ability of the SP2/mIL-6 and SP2/0 fusion partner cell lines to form stable hybrids with human splenic B cells. Expression of mIL-6 was not able to overcome the instability resulting from the segregation of human chromosomes (Dessain et al. 2004; K. Rybinski, S. Adekar, B. Barnoski, S. Dessain, unpublished data).

We originally considered that ectopic expression of human telomerase (hTERT) may improve fusions between human B cells and human immortal fusion partner cell lines. Human/human hybridomas are affected by poor proliferation rates and chromosome loss, both phenotypes having been associated in other cell culture systems with telomere dysfunction (Olsson et al. 1983; Counter et al. 1992; Bailey and Murnane 2006). Prior to the discovery of hTERT, experiments had shown that mortal human T cells impose a dominant senescence program when fused to immortal human cells (Pereira-Smith et al. 1990). Later, microcell fusion experiments revealed that the introduction of an intact copy of human chromosome 3 into an immortal, hTERT-expressing cell line repressed hTERT activity and caused cellular senescence (Oshimura and Barrett 1997). Together, these results suggested that human/human hybrid cells, formed between immortal fusion partner cell lines and primary human B cells, suffered from hTERT deficiency. Unfortunately, initial experiments with human fusion partner cell lines suggested that their deficiencies were multifactorial and could not be overcome solely by ectopic hTERT expression (S. Dessain, R. Goldsby, R. Weinberg, data not shown).

Because murine fusion partner cell lines are much better at forming hybrids than most human fusion partners, we performed similar experiments with the SP2/0 cell line (Shulman et al. 1978). The SP2/0 cell line is a very poor fusion partner for primary human B cells, so it served as a useful starting point to assess the affect of ectopic gene expression (Jessup et al. 2000). In murine/human hybrid cells, hTERT could potentially contribute to hybrid cell stability by a species-specific stabilization of human telomeres. In addition, hTERT has been shown to have many other functions that may be beneficial to hybrid cells, including an incompletely characterized tumor-promoting function that may be related to its antiapoptotic and growth factor-stimulatory activities (Holt et al. 1999; Stewart et al. 2002; Kanzaki et al. 2003; Smith et al. 2003). We found that most heterohybridoma cells formed between the SP2/0 cell line and primary human B cells expressed murine TERT (mTERT), but not hTERT. We introduced hTERT into SP2/0 cells, observing a modest increase in the numbers of cells expressing hTERT, but without useful, long-term maintenance of human antibody expression (S. Dessain, R. Goldsby, R. Weinberg, data not shown). In contrast, the ectopically expressed combination of hTERT and mIL-6 readily enabled the creation of stable hybrid cells secreting human antibodies. Notably, the hybridomas that resulted from these fusions contained considerable numbers of intact human chromosomes, even after 3 months of continuous culture in vitro (Dessain et al. 2004). An example of this is shown in Fig. 1, the human

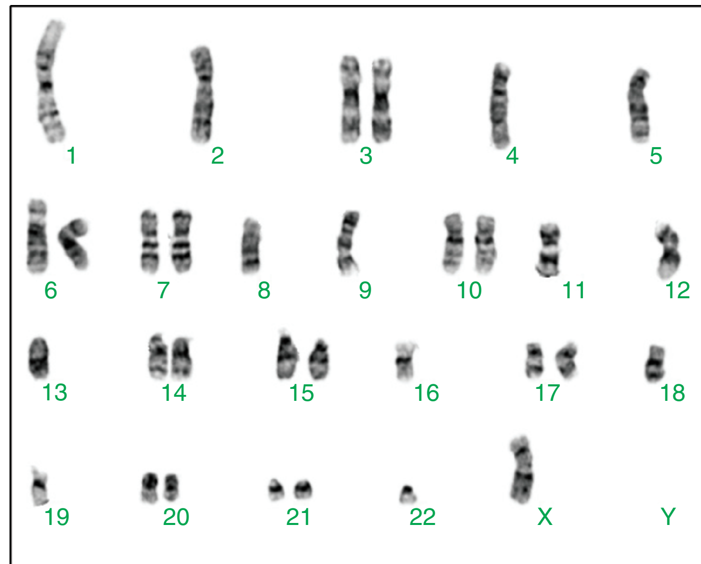

Fig. 1 Human chromosomes in a human/murine heterohybridoma. Shown are G-banded human chromosomes in a hybridoma that secretes a human antibody. Murine chromosomes are not shown. Stable antibody expression results from the ability of the hybrid cells to maintain intact human chromosomes

chromosome karyotype of a human/murine hybridoma cell that secretes a nonneutralizing IgM antibody specific for the vaccinia virus A27L antigen. Virtually a full diploid human genome is present, in addition to over 100 murine chromosomes, 10 weeks following the creation of the hybrid cell.

Some of the apparent cooperative benefit of the hTERT and mIL-6 genes may result from a mechanism whereby mIL-6 expression may promote the maintenance of human chromosomes by nascent hybridoma cells. Hybrid cells created by SP2/0 cells and human primary B cells do not proliferate in culture because the human chromosome 21 represses mIL-6 expression by the hybrid cells (Ebeling et al. 1998). Therefore, proliferation of the hybrid cells in vivo may only be possible after this chromosome is lost, indirectly selecting for cells that rapidly segregate human chromosomes. The specific mechanisms whereby mIL-6 and hTERT collaborate in chromosome maintenance are under investigation.

8 High-Throughput Screening Technologies

Following their establishment through cell fusion and drug selection, hybridomas need to be screened for specific antibody expression. In an optimal approach to thoroughly explore the native antibody immunome, each hybridoma would be

assayed individually for the binding specific of its particular antibody. Because of practical considerations, hybridomas are generally assayed in pools of dozens or more clones, but advanced screening technologies may significantly increase the yields of specific antiviral antibodies that could be obtained. New antibody screening methods differ in how they achieve the core objective of associating individual cells with the antibodies they produce. In the selected lymphocyte antibody method (SLAM), primary B cells are cultured in the presence of complement and sheep red blood cells (SRBC) conjugated to the antigen of interest (Babcook et al. 1996). B cells expressing antibodies specific for the SRBC-conjugated antigen can be identified because their secreted antibodies cause localized hemolytic reactions. Although it was originally conceived that this method would be used with primary B cells from which immunoglobulin genes would be directly cloned by RT-PCR, this method may be useful for screening hybridomas or EBV-transformed cells.

Three methods combine the isolation of individual hybridoma cells with fluorescent assays for antigen binding. The first distributes individual hybridomas into tiny wells (0.1–1 nl volume) created on glass slides using a microengraving technique (Love et al. 2006). The secreted antibodies are captured for analysis by sandwiching the arrayed hybridoma supernatants with a capture slide that is coated with secondary antibody or antigen. The bound complexes are then detected with fluorescently labeled antigens or secondary antibodies, respectively. Multiple capture slides can be used with a single hybridoma microarray, enabling cells to be screened for a variety of antigen-binding specificities. An alternate method immobilizes hybridomas on a filter through which secreted antibodies diffuse and then bind to a plate coated with a secondary antibody (www.trellisbio.com; Potera 2005). The plate is then probed with a panel of fluorescent probes that can be used in a combinatorial fashion to allow simultaneous screening for many different antigens. Computerized microscopy is used to analyze the binding reactions. Hybridomas can also be enveloped in an agarose matrix that captures the antibodies secreted by the hybridomas. For this purpose, secondary antibodies are attached to the agarose through a biotin-avidin bridge (Gray et al. 1995). The secreted antibodies are thus stably associated with the cells that produce them. The porous agarose matrix enables the hybridomas to be screened for binding to fluorescently labeled antigens. The matrix also offers structural stabilization for the hybridomas so that they can be analyzed and sorted by FACS.

In one of our laboratories (J.B.) we have begun experiments with the FMAT 8200 Cellular Detection System. This system uses antigen-coated beads, which are mixed with hybridoma supernatants and fluorescently labeled secondary antibodies in 96-well or 386-well formats. The secondary antibodies detect specific antibody bound to the beads, thereby concentrating the fluorescence into punctate signals that are detected by a mechanized plate reader. The advantage of this method is that it enables high-throughput screening of hybridoma supernatants, but it does not provide a means of isolating single cells prior to screening. Finally, the marriage of such cell screening technologies with automated cell manipulators (ClonePix, www.genetix.com) will accelerate the process of mining the native human antibody immunome to obtain antibodies for use in the treatment of viral diseases.

9 Summary and Future Prospects

The pressing demand for antibodies for use as antiviral therapeutics mandates a broad-based effort that utilizes all of the available antibody cloning technologies in parallel. Cloning methods that directly immortalize B cells through hybridoma creation or EBV infection can complement recombinant DNA and transgenic mouse methods of antibody cloning because they have an unbiased access to antibody repertoires in their native configurations. In addition, these methods simplify the exploration of the antibody repertoires of genetically diverse individuals. Over the past 27 years, successive technical advances have improved the methods for cloning native human antibodies such that they now may be able to contribute meaningfully to ongoing efforts with phage display and transgenic mouse methods. The simplicity of these methods should facilitate their application by laboratories with a diversity of research interests, as well as provide a rationale for creating core facilities that provide high-throughput screening services to academic and other researchers.

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