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Human monoclonal antibodies by immortalization of memory B cells

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The administration of hyper immune sera to prevent or treat life-threatening infections is a remarkable milestone in medicine and biotechnology that has been achieved more than a century ago. Yet, the therapeutic use of monoclonal antibodies in this field has developed slowly over the last decades. Here we compare and contrast current methods to generate human monoclonal antibodies and highlight the advantages of exploiting the human antibody repertoire using a novel method that allows efficient immortalization and cloning of human memory B cells. This method, which has been successfully applied to isolate broadly neutralizing antibodies against SARS and H5N1 influenza viruses, is expected to accelerate the development of therapeutics in the field of infectious diseases not only by providing neutralizing antibodies for passive serotherapy, but also by generating relevant information for vaccine design.

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Current Opinion in Biotechnology 2007, **18**:523–528

This review comes from a themed issue on
Pharmaceutical biotechnology
Edited by Rino Rappuoli and Jeffrey Ulmer

Available online 11th December 2007

0958-1669/$ – see front matter
Published by Elsevier Ltd.

DOI 10.1016/j.copbio.2007.10.011

Serotherapy: infectious diseases and beyond

Polyclonal immunoglobulins (Igs) isolated from hyper immune sera of animal or human origin can confer immediate protection against pathogens or toxins. This practice, introduced by von Behring and Kitasato in 1901 [1], has been extensively used in a prophylactic or therapeutic setting [2,3]. However, polyclonal sera have several problems, such as the use of heterologous proteins, the difficulty of finding immune donors and the risks related to the use of human blood products. Monoclonal antibodies represent the ideal alternative to polyclonal sera, but methods to produce such antibodies have been available only in recent years and their development in the infectious disease field has developed slowly. Indeed, currently there is only one monoclonal antibody on the market to prevent respiratory syncytial virus (RSV) infection in newborns [4].

In recent years, with the development of the monoclonal antibody technology, serotherapy has found novel applications in the field of chronic inflammatory and autoimmune diseases and in cancer. The most striking examples are provided by antibodies that neutralize inflammatory cytokines such tumor necrosis factor (TNF) in autoimmune diseases [5] and by antibodies that enhance T cell responses against cancer cells by targeting the inhibitory receptor cytotoxic T-lymphocyte antigen-4 (CTLA-4) [6] or that deplete transformed or autoreactive B cells by targeting the CD20 molecule [7]. Currently 20 humanized or fully human monoclonal antibodies are on the market and many more are at different stages of development.

In spite of the slow progress, there are several reasons to believe that human monoclonal antibodies will find relevant indications in the field of infectious diseases [8,9]. Indeed, there is an urgent need for therapies against infectious agents for which vaccines and conventional therapies are lacking. The indications range from emerging pathogens such as H5N1 influenza virus, SARS coronavirus, West Nile and Dengue viruses, to bioterrorism agents such as Smallpox and Anthrax, and to established human pathogens, such as Cytomegalovirus (CMV), HIV-1, hepatitis C virus (HCV) and hepatitis B virus (HBV), or bacteria causing nosocomial infections in immune suppressed patients. Several examples from the past and from recent studies document the effectiveness of serotherapy against some of these infectious agents both in animal models and in humans. There is evidence that sera collected from convalescent donors conferred protection against the 1918 pandemic influenza and from the emerging H5N1 virus [10,11]. Polyclonal immunoglobulins from CMV-immune donors protect the fetus when given to mothers infected with CMV [12] and HBV hyperimmune globulins can prevent reinfection of transplanted liver [13]. Monoclonal antibodies have been shown to prevent infection of mucosal tissues in a monkey model of HIV infection [14]. Finally, a low dose of a single monoclonal antibody to West Nile virus has been shown to be sufficient to protect mice from infection, while a higher dose of the same antibody can cure mice that are already infected and harbor the virus in the brain [15•].

Human monoclonal antibodies against infectious agents represent an attractive option since they have high specificity and long half-life, they lack side effects and can

synergize with antiviral and antimicrobial therapies. Furthermore, while polyclonal sera have to be given in high amounts by repeated intravenous infusions, monoclonal antibodies could be administered by intramuscular injections of much smaller volumes. While the clinical experience indicates that a single antibody to RSV is sufficient to prevent infection in newborns [4], there are reasons to believe that a cocktail of at least two monoclonal antibodies recognizing non-overlapping epitopes may be effective in preventing selection of escape mutants. This may be necessary for highly variable viruses that establish chronic infections such as HIV-1 and HCV.

Given the above considerations, it is clear that the bottleneck in the development of monoclonal antibodies as therapeutics for infectious diseases rests on the availability of high throughput methods to select potent and broadly neutralizing antibodies. The source of such antibodies and the methods used for their isolation will be considered below.

Sources of human monoclonal antibodies

In principle human monoclonal antibodies can be isolated from three sources: from immune individuals, from immunized mice or from synthetic antibody libraries. There are advantages and disadvantages in each of these approaches that may guide the choice of the most appropriate method for a given target (Table 1).

The isolation of antibodies from immune donors offers the advantage of fully exploiting the strength of the human antibody response to a human pathogen. Indeed, human antibodies have peculiar features such as long CDR3 and, being selected from a large number of precursors, can target multiple epitopes with high affinity. More importantly, the human immune system is exposed to all the relevant antigens displayed by an infecting agent and can consequently mount a broad response to most, if not all, these epitopes. This point is best illustrated by the fact that the few HIV-1 broadly neutralizing monoclonal antibodies available today have all been isolated from B cells of infected individuals [16••]. In this context, it is worth emphasizing that for any vaccine or pathogen there is a very broad spectrum of responses in the human population and it is consequently possible to identify donors that make a particularly effective antibody response, as shown recently in the case of HIV-1 [17••]. While the human source appears ideal to isolate antibodies to human pathogens, it is not in principle suitable to isolate antibodies to human antigens such as cytokines or cell surface molecules to which the human immune system is tolerant, although there may be exceptions to this rule [18].

Mice that carry human immunoglobulin loci (Hu-Mice) produce human antibodies in response to immunization and, being mice, can readily produce antibodies to human antigens [19]. Following appropriate immunization human monoclonal antibodies can be isolated using the classical hybridoma technology or alternative methods. The mouse system is therefore particularly suitable for the isolation of antibodies specific for human antigens such as cytokines or cell surface molecule but is less suitable for human pathogens such as HIV, HCV, HBV, or CMV, that do not infect mice. In addition, the immune response of these mice is often suboptimal, possibly because of the mismatch between human Ig and mouse Fc receptors.

Random synthetic libraries of human immunoglobulin (Ig) genes displayed on phages or yeasts has become a standard method to isolate human monoclonal antibodies since it can be adapted to any antigen and can lead to antibody of very high affinity through repeated cycles of affinity maturation [20,21]. A significant drawback, however, is that target antigens must be known a priori since the selection is based on binding to a purified antigen and that functional assays (i.e. viral neutralization) cannot be

Table 1

Human monoclonal antibodies (hMabs): sources and methods

	Source			
	Human plasma cells	Human memory B cells	Hu-Mice	Antibody display libraries
Preferred application	Human pathogens	Human pathogens	Human antigens	Any target
Availability of source	Narrow time window after booster immunization	Readily available Selection of best donors based on serum antibodies	Immunizations required	Readily available
Preferred method to generate hMabs	Single cell PCR of Ig genes	Improved EBV immortalization	Hybridomas	*In vitro* selection
Primary screening	Binding and functional assays after expression Screening possible only at advanced stage	Binding and functional assays on culture supernatants	Binding and functional assays on culture sups	Binding assays only
Affinity maturation	Optimal for boosted responses	Fully exploited	May be suboptimal	Can be performed *in vitro*

used in the initial step of selection. Thus, by definition, this system is not suitable to identify new neutralizing targets within complex pathogens. In addition, high affinity does not necessarily translate into higher protection if the epitope recognized is not readily available on the viral spikes. Another drawback is that the antibodies isolated in *Escherichia coli* or yeasts may be expressed suboptimally in mammalian cells.

In view of the above considerations, the targeting of the human B cell response represents the most direct and effective approach to isolate neutralizing antibodies to infectious agents that can be effectively scaled up in a mammalian expression system.

The advantage of targeting human memory B cells versus plasma cells

Having discussed the opportunities offered by the human immune response, we will now consider the advantages of targeting human plasma cells versus memory B cells to isolate human monoclonal antibodies. Newly generated plasma cells secreting specific antibodies at high rate can be recovered in high numbers and purity in peripheral blood within a narrow time window at the peak of the immune response, that is 6–10 days after a booster immunization. These cells then migrate to the bone marrow where they persist as long-lived plasma cells accounting for a small proportion of the total plasma cell pool [22]. Therefore, as consequence of their biology, plasma cells are accessible in the blood only for a narrow time window that can be precisely defined in the case of booster immunization but that is difficult to predict in the case of a natural infection. In addition, this approach does not offer the possibility of selecting high responder donors. The small numbers of plasma cells that can be isolated from blood or bone marrow and the lack of appropriate fusion partners severely limit the use of the hybridoma technology. The isolation of monoclonal antibodies from plasma cells therefore relies on cloning and expression of Ig genes. This can be done using phage display libraries of scrambled VH and VL genes isolated from bone marrow plasma cells [21] and, more recently, by isolation of paired VH and VL genes from plasma cells using single cell PCR [23••]. Using the latter method, it has been possible to isolate several human monoclonal antibodies to tetanus toxoid and influenza virus from a boosted individual [23••].

Memory B cells are known to persist for a lifetime and therefore represent an ideal repository of the antibody specificities generated by an individual following infection or vaccination that can be accessed at any time after priming by just taking a small sample of peripheral blood [24]. In contrast to plasma cells, which are terminally differentiated, memory B cells retain substantial growth potential and can be immortalized by Epstein Barr Virus (EBV). The EBV-immortalized B cells secrete antibodies in large amounts so that screening to detect specific antibodies can be performed on culture supernatants not only using binding assays but also functional assays. In addition, EBV-immortalized B cells also retain expression of surface Ig and consequently can be selected for their antigen binding capacity.

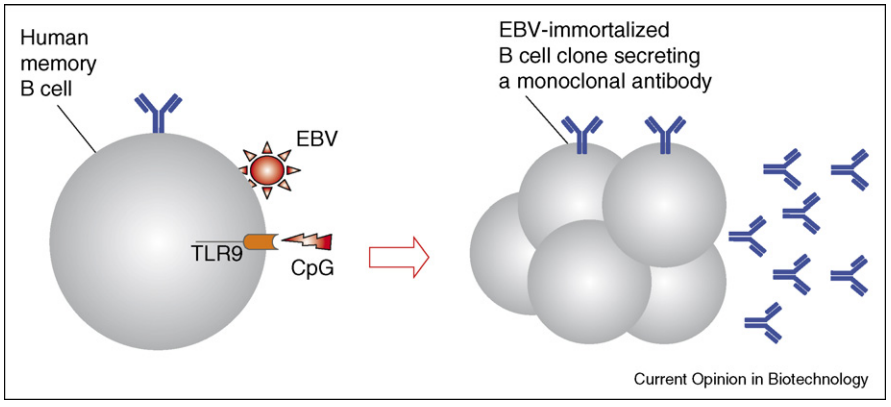
Several methods are available to purify plasma cells and memory B cells producing specific antibodies using fluorochrome-tagged antigens [25]. Antigen-specific memory B cells (including EBV-B cells) that display the specific antibody on the cell surface can be directly stained by antigen while plasma cells, that lack surface Ig, can be stained after the secreted antibody has been captured on the cell surface via a piggyback anti-Ig antibody [26]. This method is dependent on a known purified antigen and leads to the isolation of binding antibodies.

An improved method for EBV immortalization of human memory B cells

The use of EBV to immortalize human memory B cells has been reported more than 30 years ago but impact in the field has been modest because of the low efficiency of B cell immortalization and difficulties in cloning the immortalized B cells [27–29]. As a matter of fact the method was abounded in last years in favour of alternative methods such as humanization of mouse antibodies [30], selection from phage-display libraries [20,21], or immunization of mice carrying human Ig loci [19]. We recently found that addition of a TLR agonist such, for instance, the TLR9 agonist CpG, can increase more than 100-fold the efficiency of EBV B-cell immortalization and facilitate cloning of immortalized B cells [31••]. This method fully exploits the potential of the human immune response. In particular it is possible to select donors that have mounted particularly effective antibody response to the target pathogen by analyzing serum antibodies and then immortalize their memory B cells for the rapid isolation of B cell clones producing monoclonal antibodies. The antibodies secreted in the culture supernatant can be screened using both binding and, importantly, neutralization assays. In this context is worth emphasizing that not all antibodies that bind to a viral glycoprotein have also neutralizing activity, since not all epitopes are displayed on the infectious viral particle. The B cell clones isolated using the EBV method are stable and secrete antibodies at high rate (10–50 mg/L in the culture supernatants) which facilitates antibody production for preclinical studies (Figure 1).

As a proof of principle we have been able to rapidly isolate 35 monoclonal antibodies that neutralize SARS coronavirus from memory B cells of an infected donor [31••]. Interestingly, some of these antibodies have potent neutralizing activity *in vitro* and *in vivo*. Recently, four of these antibodies have been shown to neutralize all SARS

Figure 1



An improved method for immortalization of human memory B cells [31••].

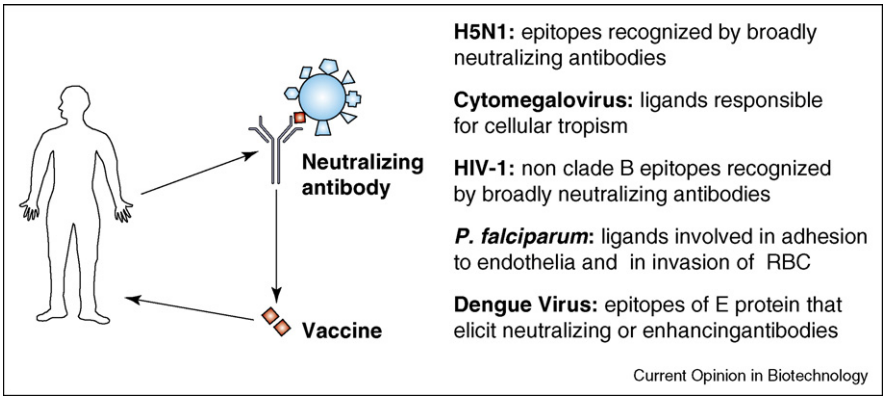
coronaviruses available including three human and two animal isolates (B. Rocks, D. Corti *et al.*, submitted). For two of such antibodies the epitopes recognized have been mapped to the receptor-binding site, while a third antibody recognizes a distinct site. Based on these findings it would be possible to formulate a cocktail of two to three antibodies that are both broadly neutralizing and recognize non-overlapping epitopes in order to provide broad protection and minimize the risk of selecting escape mutants.

In a similar process, we have isolated from a small sample of frozen PBMC from two patients recovered from H5N1 infection, several human monoclonal antibodies that neutralize H5N1 both *in vitro* and *in vivo* [32•]. Notably, these antibodies can work also in a therapeutic setting since they protect mice even when given three days after injection of lethal infectious dose. In addition several antibodies were found to neutralize both clade 1 and clade 2 H5N1 viruses.

Besides the above applications in response to emerging pathogens, the method finds is also suitable for the characterization of the antibody response against complex pathogens such as *Plasmodium falciparum*. In this case, human monoclonal antibodies have been isolated to characterize the response against VAR2CSA a molecule that mediates adhesion of infected erythrocytes to placental endothelium [33]. Other project ongoing in the authors' laboratory on HCV, HIV-1 and CMV and Dengue virus, further demonstrate the general applicability of the method.

The application of the B cell immortalization method may go far beyond infectious diseases. Indeed, several studies have shown that autoantibodies to cytokines can be produced by patients with inflammatory diseases and even by normal individuals [18] and that a small fraction of cancer patients mount an antibody response to antigens expressed by tumor cells [34]. It is possible that autoreactive antibodies isolated from such donors may find applications as diagnostics or therapeutics.

Figure 2



A schematic view of the analytic vaccinology approach and ongoing projects in the authors' laboratory. Modified from Burton *et al.* [38].

Neutralizing antibodies and vaccine design

There are two aspects of pathogens that represent a considerable challenge in vaccine design: complexity (e.g. in Herpes viruses, bacteria and parasites) and genetic variability (e.g. in HIV-1, HCV and influenza virus). A rational vaccine design rests on the identification of the molecules that promote pathogen invasion or pathology and, in the case of variable pathogens, the most conserved epitopes within these molecules. The sequencing of pathogen genomes has considerably facilitated the design of vaccines against complex pathogens through a process defined as 'reverse vaccinology', though this approach remains very labour intensive [35]. The major challenge however, is to identify conserved epitopes within highly variable viral glycoproteins.

The analysis of the human immune response and the isolation of neutralizing antibodies offer a shortcut solution to the challenge of pathogen complexity and variability. This process of 'analytic vaccinology' starts with the isolation of neutralizing antibodies that are used as probes to identify target molecules and conserved epitopes that can be then appropriately formulated as a vaccine (Figure 2). This approach has been originally proposed by Burton *et al.* [36] to solve the HIV vaccine problem and is implemented by an international consortium that works to identify conserved structure of the HIV-1 spike glycoproteins. The work has led to the identification of epitopes recognized by four broadly neutralizing antibodies, but has been substantially limited by the small number of such antibodies available. It is expected that the memory B cell immortalization method will greatly facilitate this discovery process for HIV-1 and for other complex or variable pathogens. In a reciprocal fashion, the identification of targets of disease enhancing antibodies may provide a rational approach to the design of safer vaccines that minimize the risk of immunopathology [37].

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