CHAPTER 12

Antibody Selection from Immunoglobulin Libraries Expressed in Mammalian Cells

ERNEST S. SMITH and MAURICE ZAUDERER

ABSTRACT

Vaccinex has developed an antibody discovery technology that enables efficient selection of fully functional IgG antibodies from highly diverse immunoglobulin gene libraries expressed in mammalian cells. Because antibodies are expressed and selected in mammalian cells, the Vaccinex technology avoids problems associated with alterations in the folding and activity of antibodies expressed in

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bacteria and on the surface of phage particles. Unlike mouse-based humanization and transgenic selection systems, Vaccinex's *in vitro* antibody selection technology does not suffer the limitations of immunological tolerance to highly conserved proteins. In addition, the Vaccinex technology can be used to efficiently convert nonhuman monoclonal antibodies into fully human monoclonal antibodies.

12.1 INTRODUCTION

A quarter of a century after their debut, monoclonal antibodies have become the most rapidly expanding class of pharmaceuticals for treating a wide variety of human diseases, including cancer (Adams and Weiner 2005; Reichert et al. 2005). Antibody-based therapeutics bind to and either directly or indirectly influence the activity of cells or molecules involved in a given disease. The clinical success of a number of recent antibody-based therapies such as Rituxan®, Remicade®, and Herceptin®, as well as the regulatory acceptance of antibody-based therapeutics by regulatory agencies, has caused many leading pharmaceutical and biotechnology companies to increase their commercialization focus on the development of monoclonal antibody-based therapeutics. It is estimated that 25 to 30 percent of all products currently in clinical development are antibodies.

Previously, four general strategies have been employed to produce immunoglobulin molecules for drug development. In one approach, the variable domains from rodent antibodies are isolated and cloned in frame with human immunoglobulin constant domains. This process creates chimeric antibodies that preserve the antigen-binding specificity and affinity of the original rodent antibody, but now substitute human constant domains for both enhanced effector function and reduced immunogenicity in humans (Morrison et al. 1984). Although straightforward in application, chimeric antibodies still contain on average 30 percent nonhuman sequence. The rodent variable domains often result in immunogenicity in humans.

In a related approach, rodent antibody sequences have been converted into human antibody sequences, by grafting the specialized complementarity-determining regions (CDR) that comprise the antigen-binding site of a selected rodent monoclonal antibody onto the framework regions of a human antibody (Tsurushita and Vasquez 2003). In this approach, which has been termed antibody humanization, the three CDR loops of each rodent immunoglobulin heavy and light chain are grafted into homologous positions of the four framework regions of a corresponding human immunoglobulin chain (Jones et al. 1986). Because some of the framework residues also contribute to antibody affinity, the structure must, in general, be further refined by additional framework substitutions to enhance affinity (Queen et al. 1989). Although widely employed, this technology has two limitations. First, humanization is only possible when a specific, functional, high affinity nonhuman monoclonal antibody is available as starting material. In many cases mice are tolerant to the antigen or epitope of interest. Self-tolerance makes it difficult to generate the essential murine starting material. Without this initial antibody it is not possible to undertake humanization. Second, humanization results in antibodies that are on average only 95 percent human. The remaining mouse sequences can prove to be immunogenic when injected into humans. Clearly, this immunogenicity can be disastrous for clinical development of these antibodies, and it is not possible to predict which humanized antibodies will be immunogenic short of a clinical trial.

More recently, transgenic mice have been generated that express human immunoglobulin sequences (Green 1999; Lonberg 2005). While this strategy has been successfully applied to select human antibodies to multiple target antigens, it shares with the antibody humanization approach the limitation that antibodies are selected from the available mouse repertoire which has been shaped by proteins encoded in the mouse genome. This biases the epitope specificity of antibodies selected in response to a specific antigen. For example, immunization of mice with a human protein for which a mouse homolog exists might be expected to result predominantly in antibodies specific for those epitopes that are different in humans and mice. These may, however, not be the optimal target epitopes.

An alternative approach, which does not suffer this same limitation of self-tolerance, is to screen recombinant human antibody fragments displayed on bacteriophage, yeast, or bacterial cells

(de Haard et al. 1999; Feldhaus et al. 2003; Hoogenboom 2005; Knappik et al. 2000; Marks et al. 1991; Mazor et al. 2007; Osbourn, Groves, and Vaughan 2005; Sheets et al. 1998). The most widely employed of these approaches is phage display. In phage display methods, functional immunoglobulin domains are displayed on the surface of a phage particle which carries polynucleotide sequences encoding them. In typical phage display methods, immunoglobulin fragments, for example, Fab or scFv, are displayed as fusion proteins, that is, fused to a phage surface protein. Antibody fragments with the desired binding specificity are then isolated by panning on antigen-coated tubes or by fluorescence activated cell sorting (FACS). Although this strategy does not suffer from an intrinsic repertoire limitation, it requires that variable domains of the expressed immunoglobulin fragment be synthesized and fold properly in bacterial cells. Many antigen-binding regions, however, are difficult to assemble correctly as a fusion protein in bacterial cells. In addition, the protein will not undergo normal eukaryotic posttranslational modifications. As a result, this method imposes a different selective filter on the antibody specificities that can be obtained.

As described above, each of the predominant platform technologies being used today has inherent limitations that can interfere with successfully selecting antibodies with the desired specificities or affinities. There was a need, therefore, for an alternative method to identify immunoglobulin molecules from an unbiased immunoglobulin repertoire that can be synthesized, properly glycosylated, and correctly assembled in mammalian cells.

12.2 IMMUNOGLOBULIN EXPRESSION LIBRARIES CONSTRUCTED IN A POXVIRUS VECTOR FOR EXPRESSION IN MAMMALIAN CELLS

In the following sections, we describe the Vaccinex ActivMAb® technology for selection of fully human antibodies from immunoglobulin gene libraries constructed in a poxvirus vector and expressed in mammalian cells. Different embodiments of this technology are based on construction and expression of either secreted or membrane-associated antibody libraries and support either *de novo* selection of a novel antibody, affinity improvement of an existing human antibody, or conversion of a mouse antibody to a fully human antibody specific for either soluble or membrane-associated target antigens. The principles of library construction will be presented first followed by applications of the secreted antibody platform to *de novo* selection, affinity improvement, and conversion of mouse antibodies. The advantages and limitations of the membrane antibody platform for these same applications will then be discussed.

12.2.1 Poxvirus Vectors

The ease of cloning and propagation in a variety of mammalian host cells has led to the widespread use of poxvirus vectors for expression of foreign proteins and as delivery vehicles for vaccine antigens (Moss 1991). A poxvirus-based library vector would have several advantages relative to more common plasmid or retrovirus-based vectors. Poxvirus replicates and is packaged into fully infectious particles in the cell cytoplasm and, as a result of its high infectivity, specific recombinants can be readily recovered even from very small numbers of selected cells, perhaps as few as a single cell. In addition, unlike plasmid- or retrovirus-based vectors, recombinant genes in a poxvirus vector can be recovered efficiently even from cells that have ceased to divide or that have died as a result of expression of the selected recombinant gene. Generally, the target protein coding sequence is cloned under the control of a vaccinia promoter in a plasmid transfer vector. The promoter and insert are flanked by sequences homologous to a nonessential region in the poxvirus, often the thymidine kinase (*tk*) gene, so that the plasmid intermediate can be introduced into the viral genome by homologous recombination at that locus. Recombinant virus can then be recovered based on a tk negative phenotype or other selectable marker. The frequency of recombinants derived in this fashion is of the order of 0.1 percent. This is sufficient to recover recombinants of a specific DNA clone but far too low to permit construction of a large, representative cDNA library. Initially we attempted to generate diverse

cDNA libraries in poxvirus employing a direct ligation method (Merchlinksi et al. 1997). Although this method did select for a higher frequency of recombinants, relatively low viral titers were generated. We have developed a more efficient recombination method, termed Trimolecular Recombination, which generates recombinant vaccinia virus at high frequency.

12.2.2 Trimolecular Recombination

The rationale for this strategy is that a high frequency of recombinants would be obtained if cells were transfected with defective vaccinia DNA that could be packaged into infectious particles only if it had undergone recombination. One way to accomplish this is to cut the vaccinia DNA in the middle of the *tk* gene. Since there is no homology between the two *tk* gene fragments, the two vaccinia arms cannot be linked by homologous recombination except by bridging through the homologous tk sequences that flank the insert in a recombinant transfer plasmid. Because naked vaccinia DNA is not itself infectious, production of infectious particles requires that transfection be carried out in cells that are infected with a helper virus. As previously described, fowlpox virus (FPV) does not productively infect mammalian cells but provides the necessary helper functions required for replication and packaging of mature wild-type or recombinant vaccinia virus particles (Scheiflinger, Dorner, and Falkner 1992; Somogyi, Frazier, and Skinner 1993).

A vaccinia vector, v7.5/tk, was constructed that incorporates the early/late 7.5 k vaccinia promoter as well as unique NotI and ApaI restriction sites downstream of the promoter (Merchlinksi et al. 1997). Digestion with NotI and ApaI restriction endonucleases gives rise to two large fragments approximately 80 kilobases and 100 kilobases in size. Each of these arms includes a nonoverlapping fragment of the *tk* gene for bridging by a transfer plasmid. FPV-infected cells triply transfected with the two vaccinia arms and with DNA from a cDNA library constructed in a transfer plasmid gives rise to infectious vaccinia virus that is almost 100 percent recombinant (Smith et al. 2001). This method works very reliably and has been used to construct vaccinia cDNA libraries from many different cell lines and normal tissues (Smith, Shi, and Zauderer 2004).

12.2.3 Antibody Libraries Expressed in Mammalian Cells

The Vaccinex human monoclonal antibody discovery platform is based on the monoclonal expression of recombinant antibodies in mammalian cells. Separate libraries of human heavy and light chain immunoglobulin variable genes have been constructed in a vaccinia virus-based vector by Trimolecular Recombination.

Plasmid vectors incorporating constant regions of human heavy chain secreted gamma 1, human heavy chain membrane bound gamma 1, or human light chain were constructed to accommodate cloning human Ig variable region gene segments (VH, Vκ, and Vλ) in frame. These vectors are based on the plasmid pH5/tk, in which a vaccinia early/late promoter and multiple cloning sites were inserted into the viral *tk* gene. The multiple cloning sites were modified appropriately in order to clone in a modified Ig secretory signal peptide and the constant regions of γ1-secreted or γ1-membrane immunoglobulin heavy chains, and κ and λ immunoglobulin light chains. The resulting vectors retain unique cloning sites for inserting the VH, Vκ, and Vλ variable region genes. Throughout this manuscript Vκ and Vλ are collectively referred to as VL.

In order to take advantage of the increased diversity generated by random pairing of different heavy and light chains, we elected to construct independent libraries of heavy and light chains. The overall goal was to generate libraries of sufficient complexity that appropriate VH/VL combinations will occur at a frequency that permits efficient isolation of antibodies with a desired specificity and affinity. The independent assortment of germline V (D) and J segments, as well as the random combinatorial association of VL and VH, provides substantial diversity. Further diversification occurs during the response to antigen by the process of somatic mutation. To take advantage of all diversification processes, we have produced our libraries from four different human B-cell sources: (1) commercially obtained bone marrow-derived mRNA from large donor pools, (2) commercially available peripheral

blood B-cells isolated from cancer patients, (3) commercially available peripheral blood B-cells and bone marrow from autoimmune patients (ex. Lupus), and (4) tonsil-derived germinal center B-cells. Because heavy and light chains are randomly reassorted in our system, it is possible to generate novel specificities that are more diverse than those of the antigen-driven B-cells from which these V genes derive. Somatic hypermutation in the germinal centers and selection resulting from the disease states of the B-cell donors contributes greatly to V gene diversity.

Immunoglobulin gene libraries were first constructed in the Ig-H and Ig-L plasmid vectors described above. The plasmid libraries were then used to construct immunoglobulin gene libraries in vaccinia virus by Trimolecular Recombination. It is reasonable to assume that the more VH/VL combinations that can be screened, the more likely it is that a monoclonal antibody with the desired epitope specificity and affinity will be isolated from an antibody library. Our current libraries contain 10 million Ig-H in both secreted and membrane format, and 10 million Ig-L. This calculates to a theoretical complexity of 1×10^{14} unique combinations. This complexity exceeds the size of most phage display libraries by several orders of magnitude. This theoretical complexity, however, vastly exceeds the number of recombinant antibodies that can be screened in mammalian cells. As described below, the availability of separate heavy and light chain libraries allows for sequential selection strategies that make it possible to tap into this enormous antibody diversity without needing to sample all of the 10^{14} possible VH/VL combinations directly.

12.2.4 Expression of Recombinant Human MAb

A number of mammalian cell lines were tested to identify an optimal cell line for MAb expression. Although vaccinia virus has a wide host cell range, vaccinia does not infect CHO cells or lymphocytes with high efficiency (Baixeras et al. 1998). As a result, we focused our testing on a variety of human and nonhuman epithelial cell lines. We found that HeLa cells, which are readily infected by vaccinia virus, express relatively high levels of vaccinia encoded immunoglobulins. It has been demonstrated that vaccinia virus can productively infect CHO cells if the cowpox CHO HR gene CP77 is provided by co-infection with CP77 recombinant vaccinia virus (Ramsey-Ewing and Moss 1996). We compared MAb expression levels in Hela cells versus in CP77 complemented CHO cells and determined that the expression levels were similar in the two cell lines. Because HeLa cells do not require complementation, HeLa was selected as the production line for the majority of our antibody selection projects. It is, however, useful in some instances in which a target antigen is expressed in HeLa cells to employ alternative host cell lines for antibody selection.

12.2.5 Selection of Specific Recombinant MAb

Mammalian cells infected with the vaccinia immunoglobulin gene recombinant vectors produce fully functional, bivalent antibodies. As outlined above, we have generated Ig-H libraries in a vaccinia expression vector that encodes the secretory form of the human gamma 1 heavy chain constant region. Co-infection of cells with these immunoglobulin heavy chain gene libraries and light chain libraries results in expression, assembly, and secretion of bivalent IgG1/L antibodies, permitting screening by ELISA or other functional assay. By infecting host cells with multiplicity of infection (moi) = 1 for both Ig-H and Ig-L vaccinia recombinants, each cell is on average infected with one Ig-H and one Ig-L recombinant vaccinia virus and thus expresses a single monoclonal antibody. We have also generated Ig-H libraries in a vaccinia expression vector that encodes the membrane-bound form of the gamma 1 heavy chain constant region. Co-infection of cells with these immunoglobulin heavy chain gene libraries and light chain libraries results in expression of bivalent antibody on the cell surface. The light chain libraries are the same as those used for the secreted antibody approach. By controlling moi = 1, each cell will express an average of one antibody specificity per cell. These cells can be "stained" with fluorescent labeled antigen and specific antibody producing cells selected using a combination of high throughput magnetic bead technology and cell sorting.

12.3 SELECTION OF ANTIBODIES IN SECRETED IgG FORMAT

Cells co-infected with secretory Ig-H and Ig-L recombinant vaccinia virus secrete bivalent IgG1/L, permitting screening by ELISA or functional assay. Secreted antibody has the advantage of being a very clean and specific reagent, which is particularly important for selection of antibodies to membrane-associated target antigens (see below). However, since secreted antibody is not associated with the producing cell, the selection strategy requires that small subsets of producing cells be isolated in separate wells for subsequent cloning. An efficient strategy with quantitative parameters is outlined below.

12.3.1 *De Novo* Selection of Fully Functional Antibodies in Mammalian Cells

We employ a standardized, highly sensitive ELISA for screening antibody libraries. Although each new target possesses unique characteristics, all other reagents are well characterized. The standard screening ELISA is a direct binding assay optimized for plates coated with antigen in the range of 0.1 to 1 μg/ml. Alternatively antigen can be coated onto an ELISA plate using a capture antibody. In order to achieve reliable discrimination of specific binding, it is desirable that there be at least 100 cells producing each specific antibody in an assay well. We routinely use 100,000 Hela cells for antibody production in each well, allowing 1000 different antibody combinations to be screened per well.

For screening, we generate multiple arrays in microtiter plates of mini Ig-L and Ig-H libraries, each containing a pool of 10 or 100 individual Ig gene recombinants, by amplifying 10 or 100 plaque forming units (pfu) from the parent Ig-L and Ig-H libraries, respectively, on feeder cells (monkey BSC1 cells) in individual wells of 96-well plates. Each of the resulting minilibraries contains 10 or 100 different Ig genes at titers of approximately 2×10^6 pfu/ml.

A standard *de novo* library screen is diagrammed in Figure 12.1. The Ig-H_{100} minilibraries are screened in combination with the Ig-L_{10} minilibraries. Thus, in a single well there are nominally 1000 distinct VH/VL combinations producing 1000 antibody clonotypes. Each assay well contains approximately 100,000 cells infected at a multiplicity of infection (moi) = 1 for both Ig-H and Ig-L recombinant virus. By calculation, there are nominally 100 cells producing each clonotype per well. Each 96-well plate, therefore, corresponds to approximately 10^5 distinct clonotypes. Because the independent assortment of heavy and light chains follows a Poisson distribution, the actual yield is somewhat less than this.

Following an incubation of 72 to 96 hours to allow for MAb secretion, culture supernatants are sampled and tested by ELISA for capacity to bind antigen. Specific antigens are coated onto duplicate plates, and each supernatant is also tested for binding to a control antigen. When an antigen-binding well is identified, the specific Ig-H and Ig-L are isolated from the parent minilibrary well by limiting dilution cloning and rescreening. In this process, mini Ig-H and Ig-L libraries corresponding to positive wells are sampled from the master plate and replated in 96-well plate format at limiting dilution (e.g., 10 or 1 pfu/well) and amplified on BSC1 feeder cells, creating Ig-H_{LD} and Ig-L_{LD} plates. In parallel, the original positive wells (Ig-H_{100} and Ig-L_{10}) are amplified to create high titer stocks that contain the original diversity of Ig-H and Ig-L recombinants. After amplification the Ig-H_{LD} plate is co-infected with the amplified original Ig-L_{10} stock into Hela cells and the Ig-L_{LD} plate is co-infected into Hela cells with the amplified original Ig-H_{100} stock. Following an incubation of 72 to 96 hours to allow for MAb secretion, culture supernatants are sampled and again tested by ELISA for capacity to bind antigen. In general, this screening identifies a number of positive wells in both the Ig-H_{LD} and Ig-L_{LD} plates. Following this second round of screening, individual plaques from a positive Ig-H_{LD} and an Ig-L_{LD} well are picked and amplified using standard vaccinia virus procedures. This step generates monoclonal Ig-H and Ig-L. We generally pick 12 Ig-H plaques and 6 Ig-L plaques. HeLa cell monolayers are then co-infected with each monoclonal Ig-H and Ig-L pair (12 Ig-H × 6 Ig-L = 72 combinations). The resulting supernatants are tested for antigen binding to verify that correct pairs of Ig-H and Ig-L that encode for specific antibody were isolated. The VH and VL genes are PCR

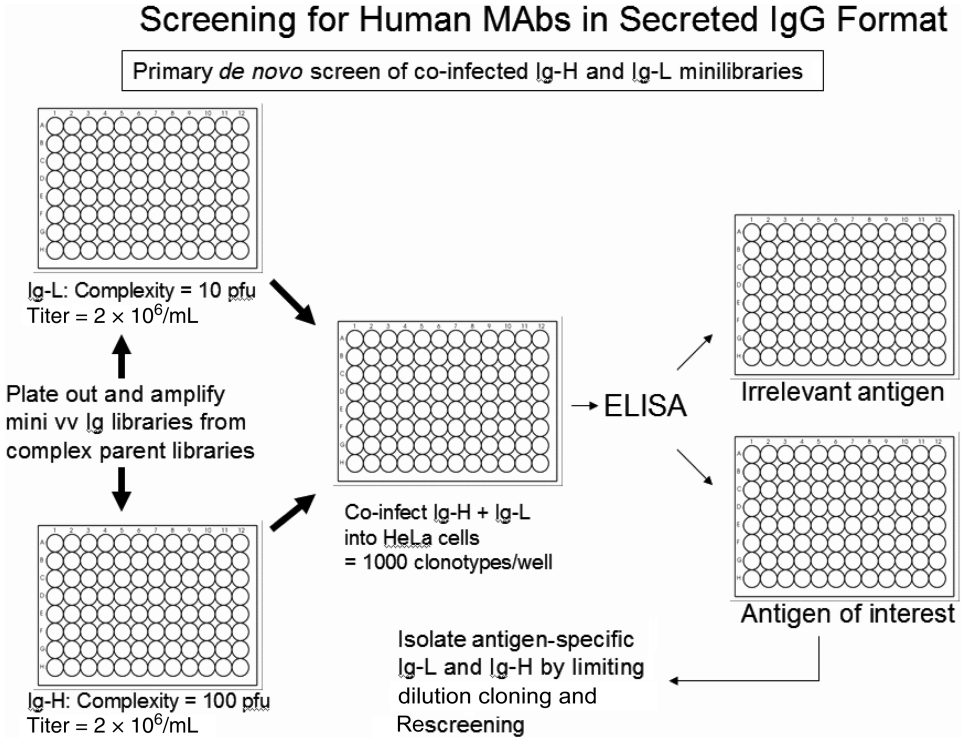


Figure 12.1 Selection of fully human antibodies using the secreted antibody platform. Separate master Ig libraries containing diverse Ig heavy and light chain genes are constructed in a vaccinia-based vector. Arrays of minilibraries of 100 Ig-H chain and 10 Ig-L chain clones are assembled and amplified from the master libraries. These minilibraries are co-infected into human cells which secrete fully assembled antibodies into the culture medium. Specific antibodies are detected by ELISA. Virus encoding the specific heavy and light chain genes are recovered from the corresponding minilibrary by limiting dilution cloning and rescreening.

amplified and subcloned into mammalian expression vectors containing human gamma 1 constant and human light chain constant domains. These expression plasmids are then transfected into CHO cells for high level expression and purification of full length IgG1/L and further analysis. Preliminary testing includes specificity testing by ELISA and affinity and functional testing.

12.3.1.1 Example of De Novo Antibody Selection Using Secreted IgG We have applied this approach to discovering MAbs to a range of different types of antigens. One such antigen, C35, is a protein of unknown function that we have determined is over-expressed in approximately 65 percent of breast tumors (Evans et al. 2006). The C35 protein can be readily expressed and purified from *Escherichia coli* as a 6X-his tagged protein. Results of a *de novo* screen for antibodies specific for C35 are shown in Figure 12.2a. The data shows ELISA results from one infection plate assayed in duplicate, as well as the same plate assayed on an irrelevant antigen. A strongly positive well was identified in this screen. The ELISA optical densities (ODs) are low but reliable. ODs increase significantly as the library complexity is reduced during second round and plaque screening. Additional screens in 200 plates identified 10 additional positive wells. The minilibraries corresponding to these positive wells were plated out at limiting dilution, rescreened as described above, and following plaque screening, pure monoclonal antibodies were obtained (Figs. 12.2b and 12.2c). The VH and VL genes from each clone were PCR amplified and subcloned into mammalian expression vectors containing human gamma 1 constant and human light chain constant domains. These expression plasmids

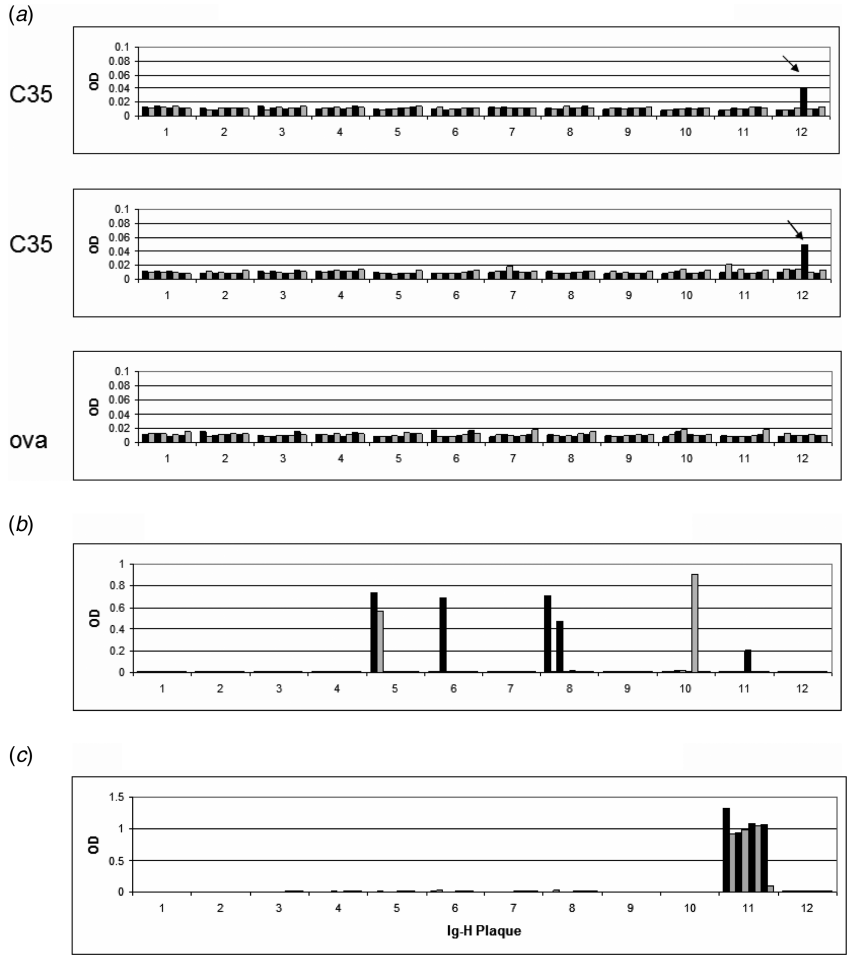


Figure 12.2 *De novo* selection of C35-specific monoclonal antibodies. Human antibodies specific for the "C35" breast cancer antigen were identified using the secreted antibody platform as diagrammed in Figure 12.1. (a) ELISA results from one 96-well infection plate. Supernatant from each well of one infection plate was assayed by ELISA for binding to C35-coated plates in duplicate, and a control antigen (ovalbumin). The well marked with an arrow indicates a well that contains a C35-specific antibody. (b) The positive well in **A** was subcloned by limiting dilution and rescreened as described in the text. A number of positive wells were identified. (c) Twelve individual Ig-H plaques were each paired with 8 Ig-L plaques. Each of the 96 plaque × plaque combinations were co-infected into Hela cells and the antibody produced was tested for binding to C35 by ELISA. The Ig-H clone is shown as a number, and each bar represents the pairing of an Ig-H clone with a different Ig-L clone. In this example, Ig-H clone 11 paired with Ig-L clones 1 to 5 created C35-specific antibodies. Sequencing of individual Ig-L clones confirmed that all five clones contained an identical Ig-L sequence.

were then transfected into CHO cells for high level expression and purification of full length IgG1/L and further analysis. Preliminary testing includes specificity testing by ELISA and affinity and functional testing.

At our current screening standard of 1000 clonotypes per well, it is possible using robotic pipeting stations (Biotek) and plate stackers for one individual to screen five million clonotypes per week. Although this number may seem low when compared to phage display throughput, we have found it to be more than adequate. In almost all cases, a workable initial panel of specific antibodies of moderate to high affinity can be isolated after screening between 2×10^7 and 10^8 clonotypes. The affinities

of these selected antibodies can vary widely. Biacore analysis indicates that the affinities of these primary antibodies are often in the 10 nM range. As discussed below, the affinity of the initially selected antibodies can easily be improved by a process of V gene replacement.

12.3.2 Affinity Improvement by V Gene Replacement Selection

Whenever necessary, affinity maturation is accomplished by a method that we term V gene replacement. In this method the antigen-specific immunoglobulin light chain and/or heavy chains are fixed and used to screen larger libraries of complementary chains (e.g., use antigen-specific Ig-L to screen larger libraries of Ig-H clones). The format for this selection is very similar to the strategy employed for *de novo* screening, except in this method one chain is fixed (Fig. 12.3a). Having separate Ig-L and Ig-H recombinant vaccinia libraries and clones greatly facilitates this approach because the antigen-specific clones are ready for V gene replacement without any further manipulation. The rationale for this approach is that during the initial selection round, the antigen-specific Ig-L was only given the opportunity to pair with 100 Ig-H clones, and the antigen-specific Ig-H clone was only given the opportunity to pair with 10 Ig-L clones. It stands to reason that if these chains are given the opportunity to pair with hundreds of thousands or millions of complementing chains, then these new pairings will create higher affinity MAbs. New higher affinity combinations are readily detected by coating ELISA plates with reduced antigen concentration. When an antigen binding well is identified, the specific Ig-H or Ig-L is isolated from the parent minilibrary well by limiting dilution cloning and rescreening as described above.

12.3.2.1 Example of V Gene Replacement A C35 specific MAb was selected with affinity of 40 nM. Affinity of this MAb was improved by V gene replacement. The Ig-L from this clone was fixed and used to screen Ig-H libraries at both the original (1 μg/ml) and reduced (0.25 μg/ml) antigen concentrations. Figure 12.3b shows the ELISA results for four new clones and the original MAb tested at these two antigen-coating concentrations. The original selected antibody did not bind strongly to wells coated with the lower concentration of antigen, while the new clones still bound as well at the lower concentration. The affinities of the new MAbs were determined to be in the low nM range. This indicated that after one round of affinity improvement clones were identified that had 10- to 30-fold higher affinity than the original MAb by Biacore analysis. Multiple rounds of affinity improvement are possible, alternating H and L chains and incorporating mutagenesis in construction of sublibraries.

12.3.3 Fixed Ig-L Screening

A number of studies have demonstrated that the antibody heavy chain often makes a greater contribution to binding than the light chain. In order to increase the throughput of heavy chain screening, we have developed a screening strategy where a small number of defined light chains are fixed, and then used to screen for heavy chains. Similar to the results of others, we have determined that a limited diversity of light chains can be used to select antibodies specific for different antigens (Merchant et al. 1998; Nissim et al. 1994). The format for this screening is very similar to that used for V gene replacement (Fig. 12.3a). Once the initial antibody is selected, the heavy chain is then fixed and used to select new optimal light chains. This approach simplifies the manipulations involved, since only one new chain is isolated, and permits more rapid screening of the Ig-H libraries.

12.3.4 Conversion of Nonhuman Monoclonal Antibodies into Human Monoclonal Antibodies

Hybridoma technology has been used to identify a number of rodent antibodies with specificity, affinity, and functional activity towards important drug targets. For drug development these antibodies are often chimerized or humanized, with the attendant risk of immunogenicity and potential loss of

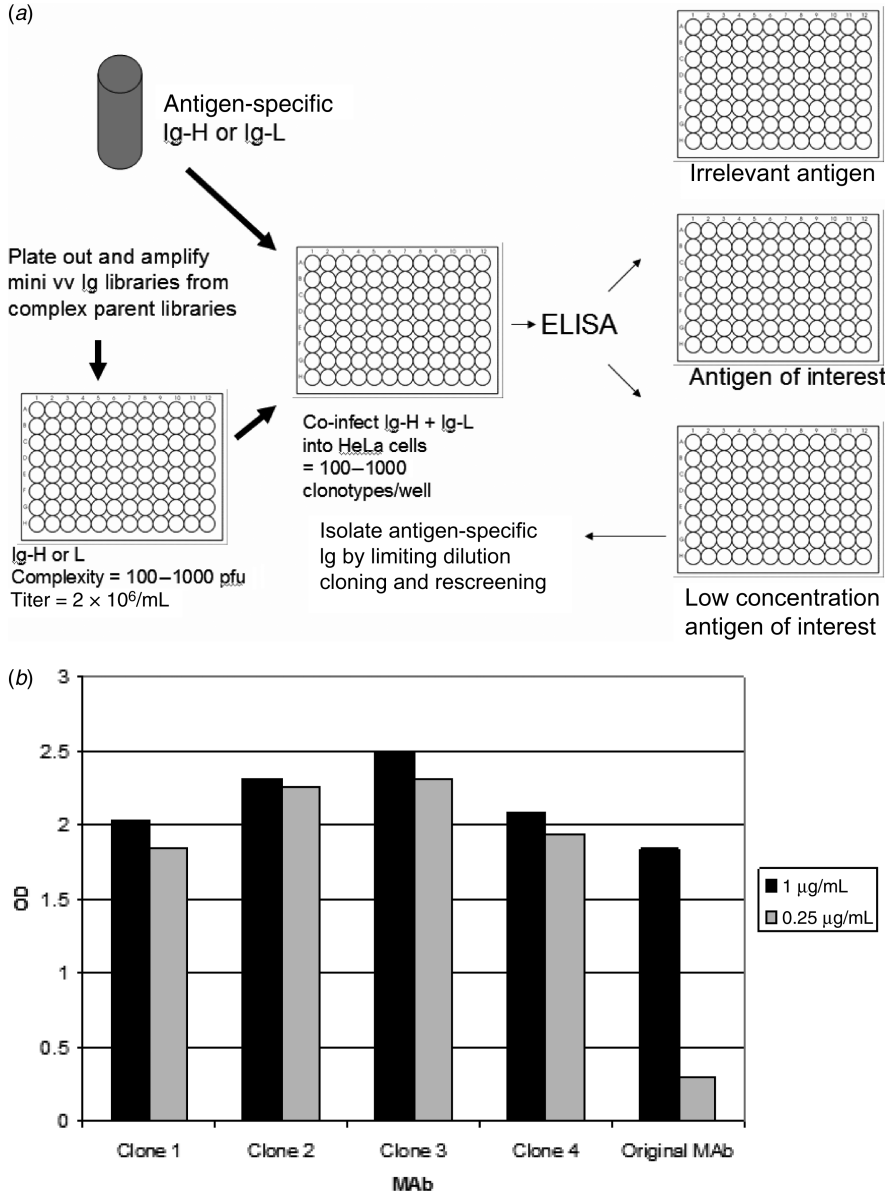


Figure 12.3 Affinity improvement by V gene replacement. (a) Affinity maturation is accomplished by pairing either the initial immunoglobulin light chain or heavy chain with a much larger selection of complementary chains. New higher affinity combinations are readily detected by coating ELISA plates with reduced antigen concentration. (b) Example of affinity improved antibodies selected by coating with a lower concentration of antigen.

affinity. We have applied the V gene replacement strategy of using vaccinia virus expressed antibody libraries for conversion of rodent antibodies into fully human antibodies.

The concept of V gene replacement in this application is to use a nonhuman antibody as a template and through a two-step process to identify human V genes that can replace the nonhuman V genes, while still retaining affinity and epitope specificity. The V gene replacement method is thus an

alternative to traditional CDR grafted humanization. This method has several advantages compared to the more traditional humanization methods:

(1) V gene replacement results in the selection of fully human antibodies, while retaining the epitope specificity of the nonhuman MAb. In principle, these antibodies should have a lower risk of immunogenicity compared to CDR grafted and framework modified antibodies that retain significant amounts of murine sequences.
(2) V gene replacement results in the selection of multiple antibodies. This allows for the selection of lead antibodies derived from distinct VH and VL germline genes with different biochemical properties, including CDR sequences, expression levels, pI, etc.
(3) V gene replacement can result in the selection of antibodies with better affinity and functional activity than the original nonhuman antibody.

12.3.4.1 Summary of the Method In the first step in the method, the V genes from the nonhuman antibody are isolated and engineered to create chimeric heavy and light chains. The nonhuman Ig-H is paired with a library of human Ig-L and screened for specific binding to antigen. This initial selection yields a panel of hybrid antibodies comprising chimeric Ig-H and human Ig-L. The selected human Ig-Ls are then paired with a library of human Ig-H and selected for binding to antigen. Parallel selections can also be carried out starting with the nonhuman Ig-L to select human Ig-H, and then using the selected Ig-H to select human Ig-L. The human Ig-L selected with the chimeric Ig-H, and the human Ig-H selected with the chimeric Ig-L can also be cross-paired. The end result of these selection strategies is that panels of human antibodies that bind to the same antigen as the original nonhuman antibody are isolated. In most cases, the selected human antibodies recognize the same epitope as the original nonhuman antibody. If necessary, the first generation human antibodies can be affinity improved through either additional rounds of V gene replacement, or through mutagenesis.

12.3.4.2 Example of V Gene Replacement to Convert IL-6 Specific Mouse MAb into Human MAbs Interleukin-6 (IL-6) is a 23 kDa protein (212 amino acids) lymphokine that stimulates both B- and T-cell functions. IL-6 is believed to play an important role in the development and progression of rheumatoid arthritis (RA) (Gabay 2006; Lipsky 2006). IL-6, in conjunction with the soluble IL-6 receptor (sIL-6Rα), has been shown to activate endothelial cells to produce inflammatory chemokines and also to upregulate expression of adhesion molecules, contributing directly to recruitment of leukocytes at inflammatory sites. In addition, IL-6 can stimulate synoviocyte proliferation and osteoclast maturation and activation, suggesting a role in synovial pannus formation and in bone resorption in the inflamed joints. Neutralization of IL-6 function by blocking its receptor (IL-6R) with an IL-6R-specific monoclonal antibody has shown considerable promise in clinical trials of patients with RA (Smolen and Maini 2006).

Preliminary clinical trials have shown that treatment of late stage cancer patients with the murine anti-IL-6 MAb BE8 can block the *in vivo* proliferation of tumor cells and reduce IL-6-related toxicities (e.g., fever, cachexia; Haddad et al. 2001; Rossi et al. 2005; Wijdenes et al. 1991). Clinical use of a murine antibody is not ideal because the murine antibodies have short half-lives in humans, requiring frequent dosing, and murine antibodies are usually immunogenic, preventing long term treatment.

We have used the vaccinia library-based antibody discovery technology to convert the murine BE8 MAb into a fully human MAb. The goal of this work was to create a human MAb that was functionally similar to BE8, but that by virtue of being human, would have a superior half-life and reduced immunogenicity compared to BE8.

A detailed description of the discovery and characterization of human anti-IL-6 antibodies will be reported elsewhere. The following is a brief description of the antibody discovery process that we followed as an example of how the vaccinia library technology is used to convert mouse MAbs into human MAbs.

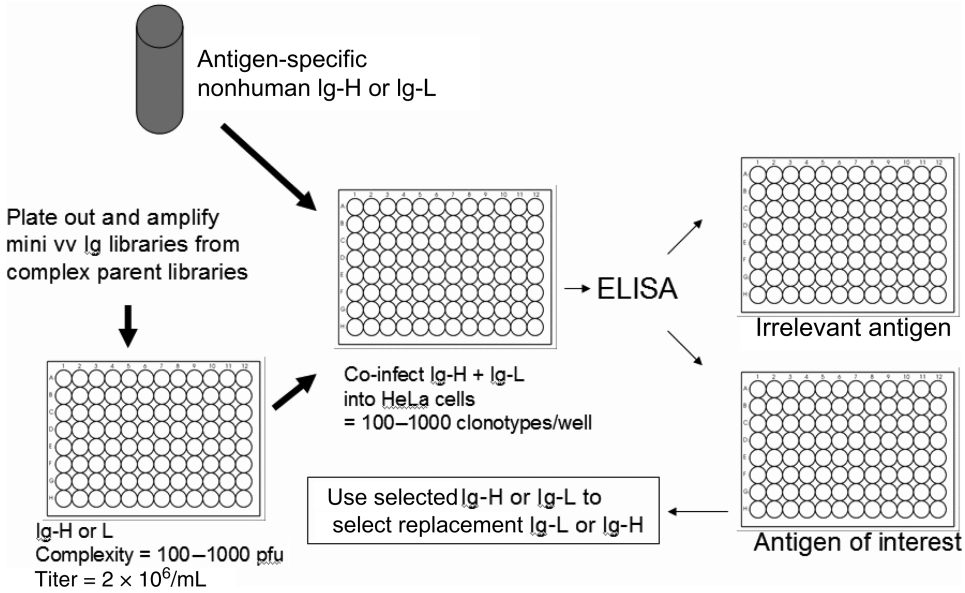


Figure 12.4 Conversion of mouse monoclonal antibody into human monoclonal antibodies. The antigen-specific mouse heavy chain is engineered so that it is expressed as a chimeric secreted heavy chain in vaccinia virus. A master Ig library containing diverse Ig light chain genes is constructed in a vaccinia-based vector. Arrays of minilibraries of 100 to 1000 Ig-L clones are assembled and amplified from the master library. The antigen-specific Ig-H and the Ig-L minilibraries are co-infected into human cells which secrete fully assembled antibodies into the culture medium. Specific antibodies are detected by ELISA. Virus encoding the specific light chain gene is recovered from the corresponding minilibrary by limiting dilution cloning and rescreening. To select human Ig-H that can pair with the selected human Ig-Ls, the process is reversed. The selected human Ig-Ls are used to screen minilibraries of human Ig-H gene libraries, each containing a pool of 100 to 1000 individual Ig-H gene recombinants. Specific antibodies are detected by ELISA. Virus encoding the specific heavy chain gene is recovered from the corresponding minilibrary by limiting dilution cloning and rescreening. Immunoglobulin genes encoding the specific antibody of interest are isolated and characterized.

The VH and VK genes from murine BE8 were engineered into full-length human Ig-H and human Ig-L in vaccinia virus so that they could be expressed as chimeric human IgG1/kappa. The chimeric BE8 Ig-H was screened in combination with human Ig-L minilibraries. A standard screen is diagrammed in Figure 12.4. For screening, we generate sets of mini Ig-L gene libraries, each containing a pool of 100 to 1000 individual Ig-L gene recombinants by amplification of 100 to 1000 pfu from the parent Ig-L libraries in individual wells of 96-well plates. Each of the resulting minilibraries carried 100 to 1000 different Ig-L genes at titers of approximately 2×10^6 pfu/mL. HeLa cells were co-infected with each of these Ig-L minilibraries along with chimeric BE8 Ig-H at moi = 1. Thus, in a single well there are nominally 100 to 1000 different Ig-L that could pair with chimeric BE8 Ig-H. Following incubation for 72 to 96 hours, culture supernatants are sampled and tested by ELISA for capacity to bind to IL-6 that had been captured onto an ELISA plate using the IL-6-specific BE4 MAb (specific for a BE8-independent epitope). When an antigen-binding well was identified, the specific Ig-L was isolated from the original minilibrary well by limiting dilution cloning and rescreening as described above. Monoclonal VL genes were then PCR amplified from the vaccinia clones and subcloned into mammalian expression vectors for high level expression and further analysis.

To select human Ig-H that can pair with the human Ig-Ls the process was reversed. The selected human Ig-Ls were used to screen minilibraries of human Ig-H gene libraries, each containing a pool of 100 to 1000 individual Ig-H recombinants as described above. We also performed parallel selections

in which we started with the chimeric Ig-L to select human Ig-H, and then used the selected human Ig-H to select human Ig-Ls.

The selected VH and VL genes were PCR amplified and subcloned into mammalian expression vectors containing human gamma 1 constant and human kappa constant domains. These expression plasmids were then transfected into CHO cells for high level expression and purification of full length IgG1/L and further analysis. Preliminary testing includes specificity testing by ELISA and affinity and functional testing. By cross-pairing all of the selected Ig-H and Ig-L, we identified over 30 human MAbs that had low nM or sub-nM affinity for IL-6.

12.3.5 Screening Antibody Libraries on Whole Cells

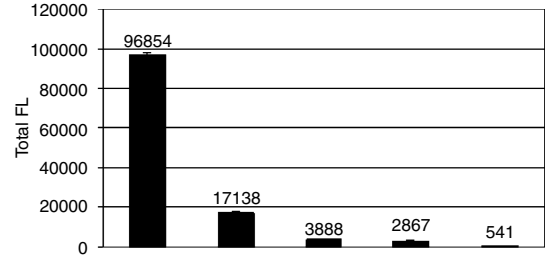
A number of multipass membrane proteins, such as G-protein coupled receptors (GPCRs), are key mediators of signal transduction (Flower 1999; Nambi and Aiyar 2003). Members of the GPCR family contain a conserved heptahelical structure that results in the proteins making seven passes through the plasma membrane. Members of the GPCR family play critical roles in numerous physiological functions and often have tissue-specific expression patterns. The critical physiological role coupled with their tissue-specific expression profile has made GPCRs attractive targets for drug development. More than 50 percent of all marketed drugs target a member of the GPCR family. Despite this high rate of development as small molecule drug targets, however, there are very few monoclonal antibodies that have been developed to target GPCRs.

The paucity of monoclonal antibodies developed to target GPCRs is mainly due to the fact that it has proven to be extremely difficult to select antibodies that are specific for GPCRs. This difficulty is due to two main factors: (1) GPCRs are hydrophobic proteins that are extremely difficult to purify in biologically active form. This requires antibody screening on whole cells. (2) Many GPCRs are highly conserved between humans and rodents and, therefore, subject to immune self-tolerance. This is an obstacle to induction of high affinity antibody responses in immunized rodents. Several approaches employing various negative and positive selection strategies have been reported for using phage display and whole cell panning for the isolation of membrane receptor-specific antibodies. Despite these isolated successes, panning of phage expressed antibody libraries on whole cells has proved to be extremely difficult. One of the main challenges results from the tendency of phage to bind nonspecifically to cells. This background binding requires precise optimization of binding and washing steps. Even when specific antibodies have been isolated by phage display, in many cases these antibodies are of very poor affinity and require extensive optimization in order to generate clinical grade antibodies.

We have developed the secreted antibody platform so as to enable screening on whole cells for the selection of fully human antibodies specific for multipass membrane proteins. This approach screens full length antibodies for binding to whole cells in the absence of interference from viral coat proteins. This approach is very similar to the approach used to screen by ELISA, except that rather than screen by ELISA, we screen using the ABI 8200 (FMAT), which was developed to allow for the screening of hybridomas that make antibodies specific for membrane antigens in mix and read format: Using this instrument a mix of soluble antibody, cells expressing the antigen of interest, and fluorescently labeled secondary reagents are added in 96-well or 384-well format, mixed, incubated, and scanned in the ABI 8200. No washing steps are required, improving throughput. With this device it is possible to analyze 60 microtiter plates in 96-well or 384-well format per day. The major limitation of this instrument is that it was designed to sample hybridomas that are present at a diversity of one antibody per well.

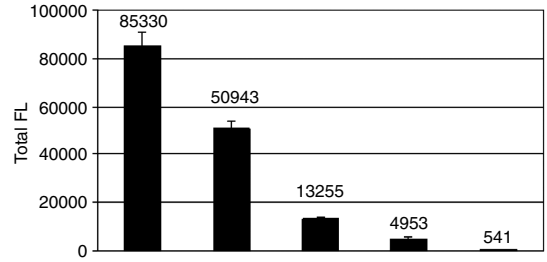
12.3.5.1 Optimization of the Platform We elected CD20 as a model system to develop this antibody selection method. CD20 is a 35,000 kDa nonglycosylated tetraspanning cell membrane phosphoprotein. The antigen is located on normal pre-B and mature B lymphocytes and is found on most B-cell non-Hodgkin's lymphomas, but is not found on stem cells, pro-B cells, normal plasma cells, or other normal tissues. This high expression on normal and malignant B-cells has made CD20 an attractive target for immunotherapeutic depletion of B-cells. Rituxan®/MAbThera®

(*a*)



MoI					
2H7 Ig-H	1.0	1.0	1.0	1.0	1.0
2H7 Ig-L	1.0	0.1	0.02	0.01	0
Control Ig-K	0	0.90	0.98	0.99	1.0
Percent CD20 Specific MAb	100	10	2	1	0

(*b*)



MOI					
2H7 Ig-L	1.0	1.0	1.0	1.0	1.0
2H7 Ig-H	1.0	0.1	0.02	0.01	0
Control Ig-K	0	0.90	0.98	0.99	1.0
Percent CD20 Specific MAb	100	10	2	1	0

Figure 12.5 Detecting secreted antibody by FMAT. The evaluation of FMAT sensitivity using vaccinia virus spiking experiments. Vaccinia virus recombinant for the 2H7 Ig-H was fixed at moi = 1, and paired with dilutions of 2H7 Ig-L recombinant vaccinia virus (a) or the 2H7 Ig-L was fixed at moi = 1 and paired with dilutions of the 2H7 Ig-H (b). In both cases the specific 2H7 virus was diluted with vaccinia virus recombinant for unselected Ig V genes. CD20-specific binding was determined by adding antibody containing culture supernatant and anti-human IgG-APC secondary reagent to wells containing CHO.CD20 or CHO.vector cells. In both cases CD20-specific antibody was detected when it was present at as little as 1 percent of the total Ig. (c) Visual examination of the wells confirms the presence of specific binding.

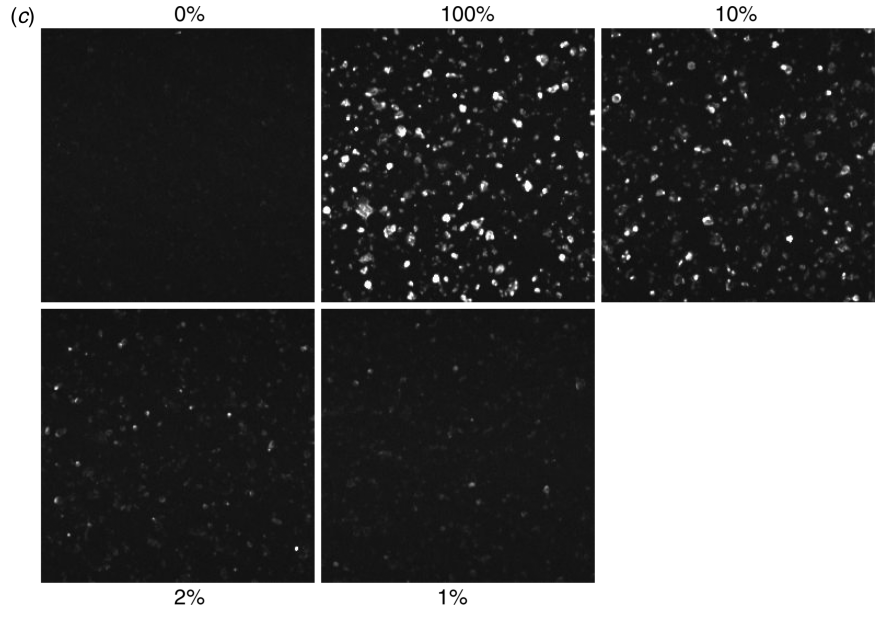


Figure 12.5 (*Continued*).

(rituximab) is licensed and commercialized in Europe and the United States for the treatment of relapsed or refractory, low-grade or follicular, CD20 antigen-positive, B-cell non-Hodgkin's lymphoma (NHL).

The mouse 2H7 MAb is specific for CD20 (Liu et al. 1987). A humanized version of this antibody is currently in late stage clinical testing (Vugmeyster et al. 2005). We cloned the CD20-specific 2H7 V genes from the 2H7 hybridoma (ATCC HB-9303) into mammalian expression vectors and into vaccinia transfer vectors for production of recombinant vaccinia virus. We also transfected CHO cells with the CD20 cDNA and selected a stable expressing clone. We used these tools: Chimeric 2H7 MAb, vaccinia virus recombinant for 2H7 Ig-H and Ig-L, and CHO.CD20 transfectants to develop FMAT conditions to detect CD20-specific Abs. Sensitivity by FMAT is a balance between signal and noise. The instrument quantitates fluorescence on each cell in a well at a specific depth of focus while subtracting out non-cell-bound background fluorescence. This enables the mix and read format because background is automatically subtracted in each well. We varied a number of conditions, including binding buffer, number of cells per well, choice of secondary reagents, binding time and temperature in order to increase the specific signal. Spiking experiments were performed to determine the limit of detection under the various conditions. In these spiking experiments, either the 2H7 Ig-H was fixed and paired with dilutions of 2H7 Ig-L, or the 2H7 Ig-L was fixed and paired with dilutions of the 2H7 Ig-H. In both cases, the specific 2H7 virus was diluted with vaccinia virus recombinant for unselected Ig-H or Ig-L genes. The data read out can be cell counts, fluorescence, or total fluorescence (counts $\times$ fluorescence) and a picture of each assay well is taken to allow for visual confirmation of positive results. As shown in Figure 12.5, in both cases we were able to detect CD20-specific MAb when it was present at as little as 1 percent of the total Ig. This translates into the ability to screen approximately 100 MAbs/well in 96 well plate format. Similar sensitivities have been observed in other antibody–antigen combinations.

Although this sensitivity limit of 100 clonotypes per well is somewhat low relative to 1000 clonotypes per well by ELISA, it is sufficient for MAb selection. We have successfully used with this approach for the conversion of mouse MAbs into human MAbs. With this approach secreted, fully human antibody can be screened on whole cells, removing the requirement for purification of

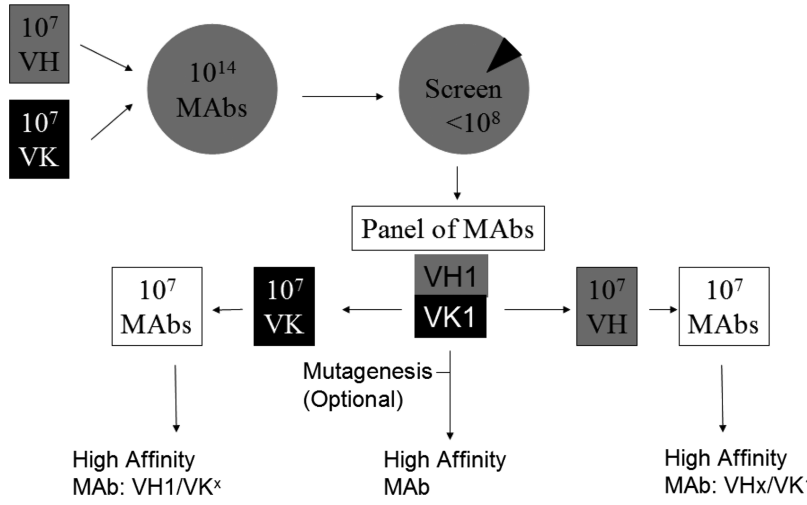


Figure 12.6 Summary of the secreted antibody platform. Employing the secreted antibody platform, 20 to 100 million recombinant antibody combinations can be screened using existing methods. By increasing automation, a larger number of antibodies could be screened. This level of diverse antibody combinations is sufficient to select a panel of fully human MAbs that have at least moderate affinity for the antigen of interest. Once an antibody of functional interest is identified, if necessary, V gene replacement screening can be employed to improve MAb affinity by rapidly sampling 10^6 to 10^7different complementary chains for both the heavy and light chains.

hydrophobic membrane proteins, and allows for the screening of antibodies against a target protein that is expressed in its natural milieu.

12.3.6 Summary of the Secreted Antibody Platform

A summary of the secreted antibody platform is shown in Figure 12.6. Employing the secreted antibody platform, it is possible to screen from 20 to 100 million recombinant antibody combinations using standard ELISA methods. By increasing automation, a larger number of antibodies can be screened. However, in our experience, this level of diverse antibody combinations is sufficient to select a panel of fully human MAbs that have at least moderate affinity for the antigen of interest and whose functional properties in the form of secreted IgG can be characterized quickly. Once an antibody of functional interest is identified, we use V gene replacement screening to improve MAb affinity by rapidly sampling 10^6 to 10^7 different complementary chains for either or both the heavy and light chains. This strategy of V gene replacement mimics the strategy of the natural immune system, which is to first select a moderate affinity antibody and then improve it. Selected MAbs can also be affinity improved by mutagenesis. This platform has been used to generate high affinity MAbs specific for a number of different types of target antigens. This technology also affords a robust method to convert mouse MAbs into human MAbs, and can be used to screen soluble antibodies for binding to membrane antigens expressed by whole cells.

12.4 MEMBRANE ANTIBODY PLATFORM

We have, in parallel, developed a platform to allow for the expression of a library of full length immunoglobulin on the surface of mammalian cells. In this embodiment the heavy chain immunoglobulin library is created in a vector that contains the human gamma 1 heavy chain containing a transmembrane domain. When cells are co-infected with heavy chain and light chain recombinant vaccinia, the antibody is expressed, assembled, and trafficked to the cell membrane. Cells expressing antigen-binding

antibodies can be isolated by staining with labeled antigen and then isolating the antigen-binding cells using a combination of magnetic bead isolation and FACS. The affinities of selected antibodies can be driven by varying antigen concentrations employed for staining and selection. This method provides a rapid quantitative method to isolate specific high affinity monoclonal antibodies.

Despite these advantages, this method of antibody selection has a number of challenges associated with it. In order to take advantage of the increased diversity generated by random pairing of different heavy and light chains, we elected to construct independent libraries of heavy and light chains. While the independent assortment of individual Ig-H and Ig-L is a great asset during V gene replacement projects, it creates additional challenges when two independent libraries must be manipulated and the selected Ig-H and Ig-L must be maintained together in order to confirm the binding properties of the resulting antibody. As mentioned above, we have found by *de novo* screening that for many antigens antibodies of at least moderate affinity occur at a frequency of between 1 in a million to 1 in 5 million clonotypes. Isolating specific cells at this frequency by MACS and FACS is extremely challenging. Because of the challenges of co-isolating independent chains and the low hit rate, we have focused our efforts with this platform on the affinity improvement of selected MAbs and the conversion of mouse MAbs into human MAbs by V gene replacement. Both of these approaches are well suited to the cell surface display strategy because when one immunoglobulin chain is fixed, the hit rate for a complementary chain increases to on the order of 1 in 100,000.

12.4.1 Conversion of Nonhuman MAbs into Human MAbs Using the Membrane Antibody Platform

The method to use the membrane antibody platform for conversion of mouse MAbs into human MAbs is shown in Figure 12.7. This follows a similar two-step process as described above for the secreted

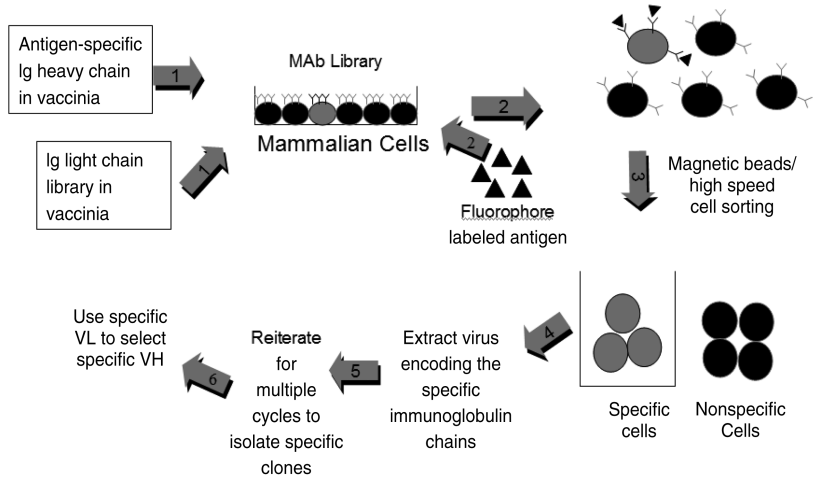


Figure 12.7 Conversion of a mouse monoclonal antibody into human monoclonal antibodies using the membrane antibody platform. The antigen-specific mouse heavy chain is engineered so that it is expressed in vaccinia virus as a chimeric membrane-bound heavy chain. Mammalian cells are co-infected with this specific recombinant heavy chain and with a recombinant library of human light chains. After overnight infection, cells are harvested and stained with fluorescent labeled antigen. Unbound antigen is washed away, and the minority of cells that express a specific antibody are isolated using magnetic bead separation and high speed cell sorting. Vaccinia virus is readily recovered from a small number of selected cells by freeze/thaw and can be rapidly expanded. If necessary initial rounds of selection can be performed until specific replacement Ig-L are identified. Once an antigen-specific fully human light chain is isolated, the chimeric heavy chain is set aside and the cycle is repeated to select complementary fully human heavy chains. This results in selection of fully human antibodies that bind the same antigen as the original mouse antibody.

antibody platform. The antigen-specific mouse heavy chain is engineered so that it is expressed as a chimeric membrane-bound heavy chain. Mammalian cells are co-infected with this specific recombinant heavy chain and with a recombinant library of human light chains. In order to simplify selection of the unknown replacement Ig-L clones, infections are performed using "inactivated" Ig-H. Inactivation is achieved by psoralen/UV treatment which blocks virus replication but permits viral entry and expression of viral and recombinant genes (Tsung et al. 1996). After overnight infection, cells are harvested and stained with fluorescent labeled antigen. Unbound antigen is washed away, and the minority of cells that express a specific antibody are isolated using magnetic bead separation and/or high speed cell sorting. This method has very high throughput. Specific antibodies can be selected from 10^8 to 10^9 recombinant antibody-expressing cells in one day. We screen our libraries at a redundancy of 100 infected cells per antibody combination, so the throughput of 10^8 to 10^9 cells translates into the ability to screen 10^6 to 10^7 Ig-L or Ig-H clones in each screening. Vaccinia virus is readily recovered from a small number of selected cells by simple freeze/thaw cycles and can be rapidly expanded by amplification on BSC1 cells. The psoralen/UV inactivated Ig-H virus will not replicate and so only the selected Ig-L recombinant vaccinia is recovered after amplification. After amplification, an aliquot of the selected pool of Ig-L can be paired with fresh chimeric Ig-H (cIg-H), stained with the antigen of interest, and analyzed by flow cytometry in order to estimate the percent of Ig-L that are able to pair with the cIg-H and bind antigen. If the percent of antigen-specific Ig-L is low, for example < 10 percent, then the pooled Ig-L from the first sort can be subjected to additional rounds of sorting and analysis until a sufficient level of enrichment has been achieved. Once the percentage of antigen-specific Ig-L is high enough, individual vaccinia plaques are picked and amplified following standard vaccinia methods. The Ig-L plaques are then paired individually with the cIg-H and analyzed for antigen binding by flow cytometry. This step identifies clonal human Ig-L that can replace the cIg-L for pairing with the cIg-H and binding to antigen. Once an antigen-specific human light chain is isolated, the chimeric heavy chain is set aside and the process is repeated using the human Ig-L to select complementary human heavy chains. This results in selection of human antibodies that bind the same antigen as the original mouse antibody. Parallel selection strategies can be carried out starting with the chimeric Ig-L to select replacement human Ig-H, and then using the human Ig-H to select human Ig-L. The selected VH and VL genes are then PCR amplified from vaccinia and subcloned into mammalian expression vectors containing human gamma 1 constant and human kappa/lambda constant domains. These expression plasmids are then transfected into CHO cells for high level expression and purification of full length IgG1/L and further analysis. Preliminary testing includes specificity testing by ELISA, affinity measurements, and functional assays. If necessary, these antibodies can be affinity improved either through V gene replacement or through mutagenesis.

12.4.1.1 Example of Conversion of Mouse MAb into Human MAb Using the Membrane Platform The 3C9 hybridoma produces a mouse MAb that binds with high affinity to the proprietary VX5 antigen. We used the membrane antibody platform to convert 3C9 into a human MAb. In the first step the V genes from the 3C9 hybridoma were isolated and used to generate vaccinia virus recombinant for membrane bound chimeric Ig-H and chimeric Ig-L Next, we co-infected Hela cells at moi = 1 with the c3E10 Ig-L and a library of human Ig-H. After overnight infection, the cells were harvested and incubated with 50 ng/ml VX5 for 30 minutes on ice. Following this incubation unbound VX5 was washed away and the cells were incubated with biotinylated polyclonal anti-VX5 antibody (pAb) for 30 minutes on ice, washed, and incubated with streptavidin-APC for 20 minutes on ice, washed, and then cells binding to VX5 were isolated by cell sorting. Virus was released from selected cells by three cycles of freeze/thaw and selected virus was amplified on BSC1 feeder cells in one well of a six-well plate. After amplification and titration, the selected virus was used as input for a second round of selection as described above. Following the second round of selection, individual Ig-H plaques were picked and analyzed for the ability to pair with c3C9 Ig-L and bind to VX5. As shown in Figure 12.8a, a panel of Ig-H clones was isolated. Preliminary characterization of hybrid antibody (cIg-L/Ig-H) allowed the selection of a panel of

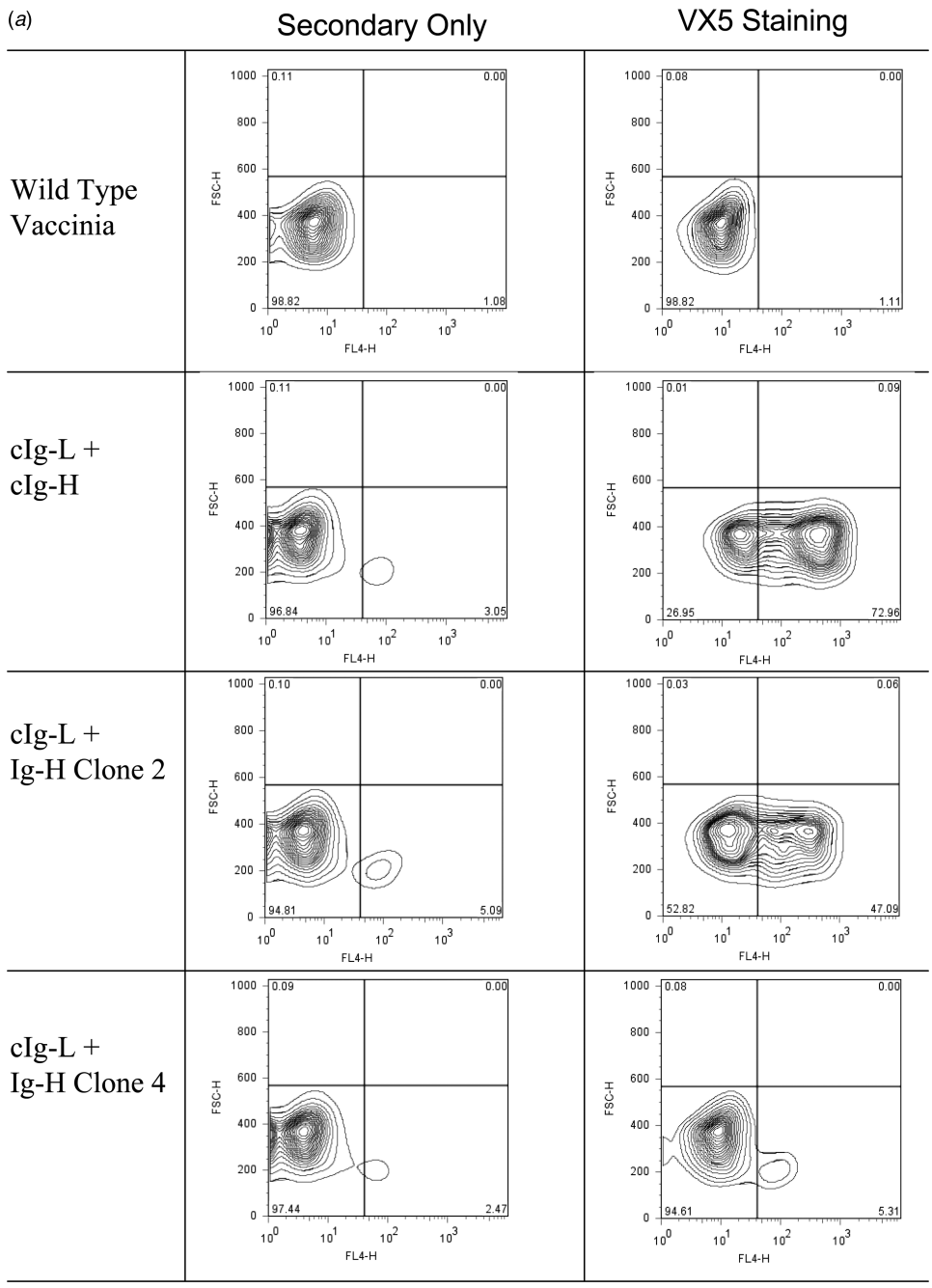


Figure 12.8 Flow cytometric analysis of antibodies selected with the membrane antibody platform. (a) Chimeric VX5 specific Ig-L was used to select replacement human Ig-H as described in Figure 12.7. Following two cycles of selection individual human Ig-H clones were tested for the ability to pair with the Chimeric Ig-L and bind to antigen. Ig-H clone 2 is a representative antigen specific clone, while clone 4 is a representation clone that does not bind to VX5.

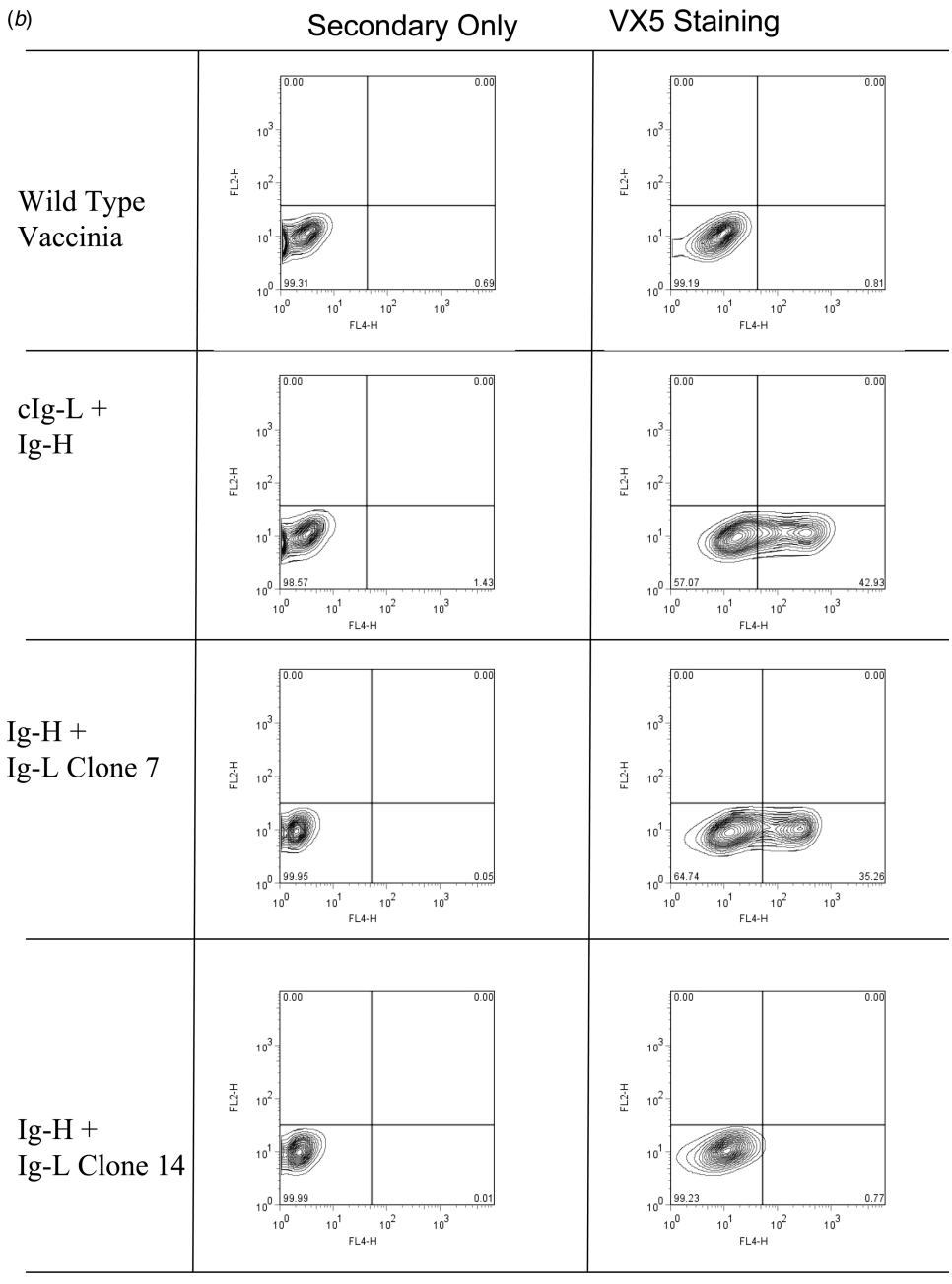


Figure 12.8 (*Continued*) (b) The human Ig-H was used to select human Ig-L. Clones were analyzed for the ability to pair with the Ig-H and bind to VX5. Ig-L clone 7 is a representative antigen specific clone, while clone 14 is a representation clone that does not bind to VX5.

promising Ig-Hs. Next, each human Ig-H was fixed and used to screen a library of human Ig-L. After two rounds of selection a panel of human Ig-L was isolated (Fig. 12.8b). The selected VH and VL genes were then PCR amplified and subcloned into mammalian expression vectors containing human gamma 1 constant and human kappa/lambda constant domains. These expression plasmids were then transfected into CHO cells for high level expression and purification of full length IgG1/Ls. Preliminary testing includes specificity testing by ELISA and affinity and functional testing. Selected antibodies had affinity (1 to 10 nM) and functional activity that was similar to that of chimeric 3C9.

12.4.2 V Gene Replacement with the Membrane Antibody Platform

The membrane antibody platform can be used to affinity improve antibodies by V gene replacement. These V genes can come from the conversion of mouse MAbs into human MAbs, from the secreted antibody platform, or from any other source such as phage display. This approach follows a similar strategy as described above for the secreted antibody platform and for the conversion of mouse MAbs into human MAbs using the membrane platform. An antigen-specific chain is fixed and used to interrogate a large number of complementary chains. The advantages of using the membrane format for affinity selection are twofold. First, this method has a very high throughput, with more than one million chains being screened at once. Second, since antibody-antigen binding is occurring in solution, affinity selection strategies have been developed where the concentration of selecting antigen is reduced from initial selection rounds to select for only higher affinity binders (Van den Buecken et al. 2003). By iterative bootstrapping with this strategy we can mimic the effect, although not the mechanism, of *in vivo* affinity maturation to isolate high affinity MAb.

12.4.2.1 Example of V Gene Replacement with the Membrane Antibody Platform Asdescribed above, we used the secreted antibody platform to convert the IL-6 specific BE8 MAb into a panel of human MAbs. One of these antibodies, MAb 190, had good functional activity in the *in vitro* assays, but had an affinity of only 6 nM. We elected to affinity improve MAb 190 using the membrane antibody platform. For affinity improvement, we fixed the Ig-L (L172) from MAb 190 and used it to screen a library of Ig-H. Because Ig-L has the same format for secreted and membrane-bound antibodies, the vaccinia clone selected with the secreted platform could be directly used for selection of heavy chain in the membrane platform. HeLa cells were infected with psoralen/UV inactivated Ig-L (L172) and a library of Ig-H. Following an overnight infection the cells were harvested and stained with 1 nM IL-6 for 30 minutes on ice. After washing, bound IL-6 was detected using the IL-6-specific B-F6 antibody which recognizes a different epitope than BE8/MAb 190. The B-F6 MAb is a mouse IgG1 MAb, and bound B-F6 was detected by staining the cells with goat-anti-mIgG1-APC polyclonal antibody. The brightest fluorescent cells were sorted, vaccinia virus was extracted from these cells by three cycles of freeze/thaw, and the selected virus was amplified on BSC1 feeder cells and titered. HeLa cells were co-infected with an aliquot of the amplified bulk sorted virus, Ig-H_1, and L172. The cells were stained for IL-6 binding as described above and analyzed by flow cytometry. As shown in Figure 12.9, a significant percentage of cells stained positive for IL-6 binding, indicating that a significant percentage of the Ig-H clones contain an IL-6-specific VH. Twenty vaccinia clones were picked from Ig-H_1, amplified, paired with L172, and analyzed for IL-6-specific binding. Eleven of the twenty clones produced an Ig-H that paired with L172 and bound to IL-6. Figure 12.9 shows representative data from several clones. The selected VH genes were then PCR amplified and subcloned into a mammalian expression vector containing human gamma 1 constant domain. The Ig-Hs were then cotransfected along with the L172 expression plasmid into CHO cells. MAb was purified and characterized for specificity, affinity, and functional activity. Affinity data demonstrated that five of these new MAbs had an affinity that was improved compared to MAb 190, with the new affinities ranging from 0.6 nM to 1.1 nM. This data demonstrates that with a single round of affinity improvement we had generated a number of new MAbs with 5- to 10-fold improvement in affinity.

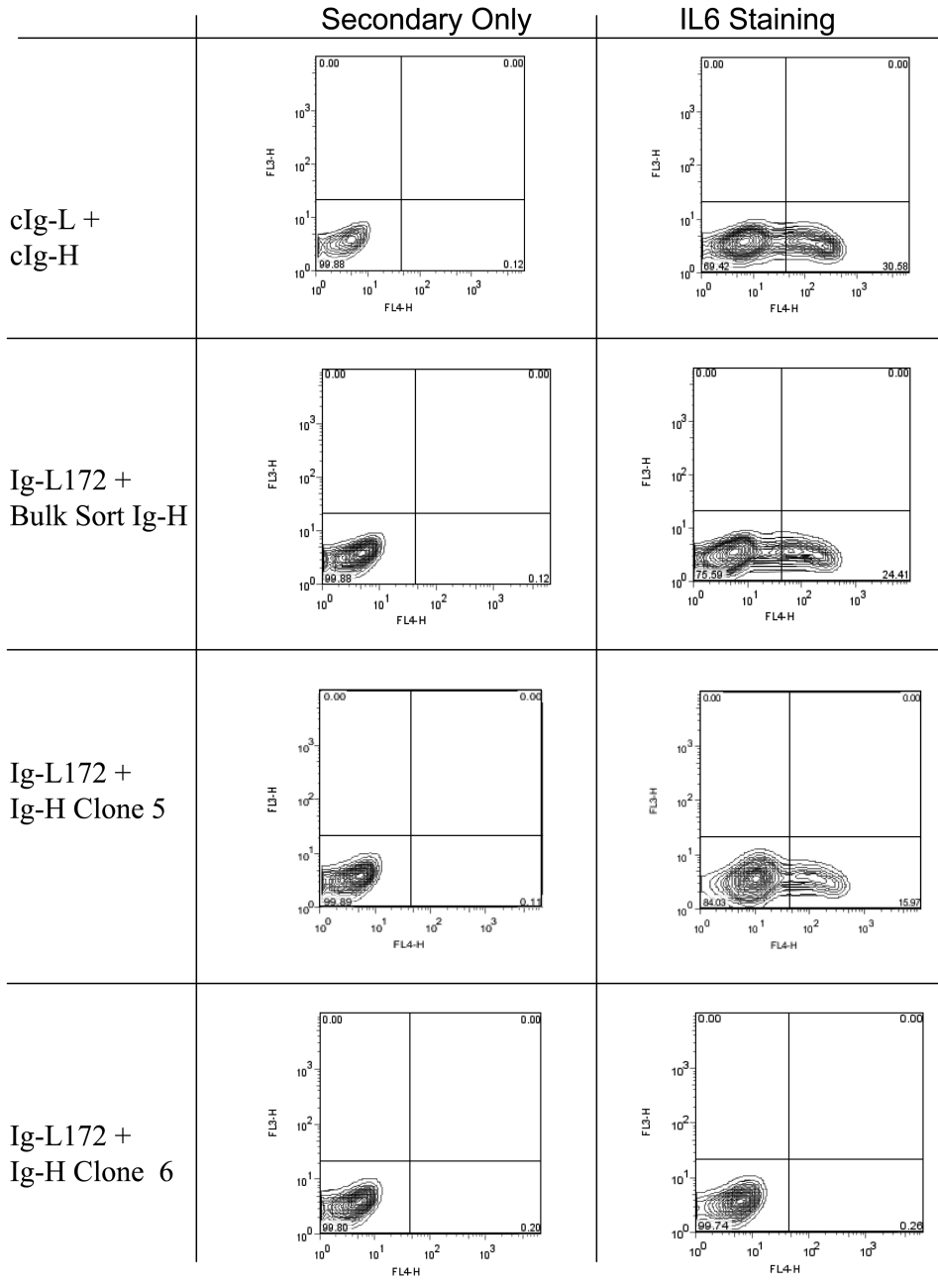


Figure 12.9 Affinity improvement by V gene replacement. An IL6 specific MAb was affinity improved by fixing the antigen specific Ig-L, and using this Ig-L to screen a library of human Ig-H. This approach is very similar to the approach outlined in Figure 12.7, except a human Ig-L is fixed instead of a non-human Ig. After one round of sorting, individual Ig-H clones were tested for the ability to pair with the Ig-L and bind to IL6. As shown in the figure, clone 5 is antigen specific, while clone 6 is not.

TABLE 12.1 Advantages of Expressing Immunoglobulin Libraries in Mammalian Cells

Existing Limitation	ActivMab Technology Advantage
Reengineering of scFv selected from phage and yeast display libraries	Engineering takes time and can create unplanned difficulties in production. Vaccinex directly expresses complete MAbs
Manufacturing	Intrinsic selection for high expression in mammalian cell lines, easily adaptable to manufacturing
Conversion of nonhuman antibodies	Many nonhuman MAbs exist, Vaccinex technology allows such antibodies to be converted into 100% human antibodies
Tolerance	Broader target range than mouse-based platforms since no homology issues
Difficult membrane targets	No interference in binding of soluble, secreted IgG to membrane targets

12.5 ADVANTAGES OF ANTIBODY SELECTION FROM IMMUNOGLOBULIN LIBRARIES EXPRESSED IN MAMMALIAN CELLS

The ability to select fully human monoclonal antibodies from immunoglobulin gene libraries expressed in mammalian cells has a number of advantages. First, using any of the variations of this technology, antibodies are selected as full length IgG antibodies. Other library technologies express and select antibody fragments, usually scFv or Fabs, which must be reengineered into full length IgG antibodies. Unanticipated difficulties can sometimes arise when these antibody fragments are removed from their selected environment and expressed as full length IgG antibodies. Second, employing this technology, there is a built in selection for antibodies that are efficiently expressed in mammalian cells. Any antibodies that do not express well will likely not be produced at sufficient concentration in the cell supernatant or on the cell surface to enable discovery with this technology. To date every antibody selected with this ActivMAb technology was efficiently expressed when transfected in CHO cells. Third, this technology has proven to be a robust method to convert nonhuman antibodies into human antibodies. Multiple potential lead antibodies are selected, and these human antibodies reproduce the affinity and functional activity of the parental murine antibody. Fourth, antibody selection with this technology is not restricted by self-tolerance. Similar to other library technologies this technology enables the selection of antibodies to virtually any target antigen. Fifth, secreted antibodies can be generated from our libraries and screened for binding to whole cells. This enables discovery of antibodies to proteins that cannot be readily purified. A summary of the advantages of this technology is shown in Table 12.1.

ACKNOWLEDGMENTS

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