Human Vaccines

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Emerging Technologies in Design and Development

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Foreword

It is a clichéd but undeniable claim that vaccines have been one of the most effective medical interventions in human history. However, the science of vaccine development has evolved considerably over the years since Pasteur created the first vaccines in his laboratory. Attenuation or inactivation of whole bacteria and viruses by physicochemical means served vaccine development well for the earliest products.[1,2] Gradually, over the course of the 20th century, an evolution in attenuation techniques took place. Pathogens were passaged in cell culture or in animals, allowing for the selection of less virulent strains. Chemical inactivation also became more precise, improving the immunogenicity of toxoids or whole organisms. Later, a common strategy was formulated to develop vaccines based on proteins or polysaccharides that elicited protective immune responses during natural infection. The advent of genetic engineering made it possible to produce large quantities of those protective antigens. These basic techniques worked well in situations where relatively straightforward antibody or cellular responses to single antigens were protective or where replicating attenuated organisms could induce protection without disease.

As we move further into the 21st century it has become clear that a deeper understanding of antigenic structure and immune responses is necessary for future success. Each pathogen presents a unique set of challenges for vaccine development, but there are common deficiencies in defining and developing immunity against all infectious diseases that can be addressed by emerging technologies. The inability, thus far, to produce highly efficacious vaccines against HIV or tuberculosis is emblematic of these deficiencies, but vaccine development against other diseases is also impeded by our collective ignorance. A prime example is that of respiratory syncytial virus (RSV), the first and second most important causes of respiratory disease in infants and the elderly, respectively.[3–6] Although an effective vaccine against RSV should produce functional antibodies, the epitopes that are important for neutralization are not present on all forms of the virus's surface fusion protein.[7] A cellular response may also be important to protection, particularly in the elderly, but we do not know whether CD4+ or CD8+ T cells are more important or if they might actually be immunopathogenic rather than immunoprotective. In addition, we do not know why natural reinfection with RSV is so frequent. To put it another way, what is the defect in natural immunity to RSV?

Another striking example of our ignorance of immune correlates of protection relates to pertussis vaccines. Neither whole cell nor acellular vaccines give long-lasting immunity, although the latter clearly gives less durable protection.[8,9] How can we generate the kind of T helper cell responses that will boost and prolong B and T cell effector memory in the case of infections with a short incubation period and central memory in setting of infections with a long incubation period. The value of effector CD8+ T cells is also becoming more apparent across various vaccines, including those against HIV.[10] When it comes to antibody responses, great strides have been made in their characterization, but there remains a great need for more detailed understanding of antibody functions. Our artificial in vitro neutralization tests do not reflect all functions of protective antibodies. For example, we know that antibody avidity and isotype influence immunity[11,12] and that non-neutralizing antibodies acting through antibody dependent cellular cytoxicity are also important to protection from disease; but none of these mechanisms are captured on our standard immunoassays.

As vaccine-elicited immunity is better elucidated, improved platforms for vaccine delivery are also going forward in parallel. Vectors, one of the more versatile vaccine platforms, are defined as microbes or nucleic acids carrying information for a pathogen protein that induces a protective response. The simplest case is a DNA plasmid coding for a surface protein such as HIV gp120.[13] More recently, RNA segments have also come into play, expressing proteins, for example, that are important for protection against cytomegalovirus.[14] Viral (e.g., adenoviruses, poxviruses) and bacterial (e.g., BCG) vectors are the most widely used, expressing specific proteins during either complete replication or a single cycle of defective replication. This strategy has been used most extensively for HIV vaccines.[15,16] The biggest challenge with vectors is the induction of immunity to the vector itself, precluding the use of multiple doses in one vaccination regimen. The way around this impediment has been through the use of regimens of heterologous vectors, the so-called prime-boost strategy. Typically, two doses of a DNA- or adenovirus-based vector are administered, followed by a poxvirus vector or the protein of interest. Even if we overcome the problem of vector immunity, we still do not understand why prime-boost regimens work so well. Confounding the issue even more is that all vectors are equally immunogenic. Adenovirus vectors tend to generate higher CD8+ T cell responses, whereas poxvirus vectors are better at generating CD4+ T cell responses.[17,18] Cytomegalovirus vectors induce CD8+ responses that vary with the genetic content of the insert.[10] This is where systems biology and basic immunology could give us crucial information to enable better understanding and use of vectors.

Among the many emerging technologies, the use of genomics, sometimes called reverse vaccinology, has great potential in offering up new antigenic targets for vaccine development. This technology has already yielded one vaccine, meningococcus Group B,[19] but there are many other bacteria and

some large viruses that could undergo genomic analysis for the purpose of identifying immunogenic epitopes. In fact, organisms with large genomes are likely to have both "genes" that induce protective immune responses and "genes" that inhibit the host from generating protective immune responses. Genomics should thus permit selection of the right mixture of antigens. Vaccines against staphylococcus, as an example, could greatly benefit from reverse vaccinology. Virus like particle (VLP) technologies have also emerged in recent decades as a powerful tool for vaccine development. The VLP human papillomavirus vaccines are among the most safe and efficacious vaccines available, paving the way for the same strategy to be applied to other viruses whose correlate of protection is associated with a single surface protein. Norovirus VLP vaccines appear to be working well; however, the organism may mutate sufficiently to require periodic updates of vaccine antigens.[20] One solution to pathogen mutability could be the use of VLPs that present an array of heterologous antigens.[21] Nanoparticle vaccines may be an attractive alternative to VLPs as they can also present multiple antigens, but enter cells more easily, including those in the intestinal tract, thus facilitating more robust mucosal immune responses.[22–24]

Notable among all the technological developments in vaccinology is the growing tendency to move away from live attenuated vaccines, largely owing to theoretical or actual safety concerns. The consequence of this trend is the need for more powerful adjuvants to nonreplicating antigens. This is an area rich in possibilities—already progressing through the use of toll-like receptor (TLR) agonists—but one that will require careful consideration of safety in general but also with specificity to the genetics of the vaccinated population, a concept that has been termed "adversomics."[25] Thus, adjuvants may have to be chosen with regard to the immunogenetic group of the vaccinee.

All of this requires that we learn much more about how to induce adaptive immune responses that are not only protective but durable. We have much to learn about the breadth and memory of B and T cell responses before we can mimic the solid protection that often follows natural infection. Indeed, in some cases like RSV and malaria, we wish to do better than natural immunity. A major collaborative Human Vaccines Project is needed to reach these goals.[26] Predicting the course of vaccinology is probably no more certain than predicting the weather. Be that as it may, I foresee that no future vaccine will be developed without a profound understanding of the structure of the antigens used. The most successful vaccine antigens will contain only those portions that generate protective responses, although vaccines to inhibit certain adverse effects such as allergic responses could also be possible if we identify the right epitopes.[27] In the coming decades, vaccines that induce durable, protective immunity will also be based on knowledge of how best to stimulate T follicular helper cells, plasmablasts, and where required, CD8 + effector T cell responses. Innate immunity elicited

by vaccination will also need be better characterized, as it will not only influence protection but have nonspecific effects on other pathogens as well.[28] All vaccines likely will need to be adjuvanted to stimulate both innate and adaptive immune responses.

There is a long and growing list of diseases for which vaccines are needed. Ultimately, the advent of new strategies and technologies to induce robust immunity bodes well for the future of vaccine research and development. However, beyond the need for advances in basic science, we must learn better ways to demonstrate safety and efficacy of vaccines with greater cost- and time-efficiency. So the vaccine enterprise has much to do in order to reap the fruits of new discoveries. This volume speaks to how we can build into on vaccinology's solid foundation of the past hundred years and recapitulate our successes in the coming century.

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Chapter 1

Broadly Neutralizing Antibodies

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The antibody response to human pathogens is generally robust, highly specific, long-lasting and, in many cases, able to clear infection. The initial encounter between a naïve B cell receptor (BCR) and a foreign antigen activates B cell clonal lineages that subsequently undergo somatic hypermutation and selection in a process that increases antibody affinity. In most cases, the first detectable antibody response in the plasma is of the IgM class, switching to IgG and IgA classes within several weeks after infection. As most high-affinity antibodies develop, B cells engage $CD4^+$ T follicular helper cells in germinal centers and exit as plasmablasts. Intermediate IgM and class-switched memory B cells are also released into circulation at predictable intervals during the process.[1,2] Large quantities of antibodies are produced by short-lived plasmablasts that are found in the circulation during an acute infection. These cells appear in particularly high numbers in response to human immunodeficiency virus type 1 (HIV-1), although the vast majority are not HIV-1-specific because of extensive B cell hyperactivation that is a hallmark of the disease.[3] For those infections that are cleared, resolution is associated with a decline in the circulating plasmablast and retention memory B cell pools that are available for recall upon subsequent exposures. Consequently, secondary responses are more rapid, generate higher affinity antibodies and mediate protection against reinfection or at least severe disease.

The BCR is an integral membrane form of the antibody that is specific to each B cell. Antibodies are heterodimeric proteins consisting of heavy and light chains that combine to form a basic "Y" shaped structure. Both surface-bound and secreted antibodies have a compartmentalized construction that includes a region able to recognize antigens. The process of antibody gene rearrangement[4] results in a large array of antibody binding sites that are further diversified by somatic hypermutation.[5] Each antibody

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contains two antigen recognition sites making up each arm of the "Y" shaped structure. These portions—the "fragment antigen binding" or Fab regions—are the primary focus of efforts to isolate and characterize human antibodies. The third major functional and structural component of an antibody is the "fragment constant" or Fc region that defines antibody isotypes and subclasses. It interacts with effector arms of the immune system either by binding receptor molecules (e.g., plasma complement proteins) or by binding cell surface receptors on effector cells (e.g., NK cells). These Fc-mediated effector functions likely play an important role in a number of infections as they enhance the antiviral efficacy of antibodies. Antibody functions can be further manipulated through recombinant engineering of the Fc region, either by mutating amino acid residues, changing glycosylation patterns, or both. This has been a common practice for the development of monoclonal antibodies (mAbs) in clinical use.

The most important antiviral function of an antibody is pathogen neutralization, mediated through the specificity afforded by the Fab portion. Neutralization is a measure of the ability of an antibody to prevent pathogen entry into a cell, and it is thought to occur by a variety of mechanisms that include steric hindrance, target dissociation and promotion of structural inflexibility in the pathogen's surface proteins. Effective neutralization is dependent on antibodies that target functionally active sites. Those antibodies that recognize highly conserved regions in the pathogen proteins are more likely to be broadly neutralizing and, therefore, most desirable to elicit when designing a vaccine.

The isolation of broadly neutralizing antibodies has been a major focus of efforts to develop vaccines against many pathogens, including HIV, influenza, respiratory syncytial virus (RSV).[6] In addition to their roles in preventing, reducing and clearing infection, neutralizing antibodies serve as a correlate of protection for most human vaccines.[7] Thus, studying the targets of protective antibodies could result in improvements to existing vaccines or the development of novel ones. Furthermore, isolation, detailed biochemical characterization, epitope mapping and structural modeling of mAbs could pave the way for the development of vaccines and therapeutics for a range of diseases for which no interventions are currently available.

IDENTIFICATION OF BROADLY CROSS-REACTIVE ANTIBODIES IN HUMAN DONORS

The detection of serum antibody responses to a pathogen of interest is generally a good indication of the presence of circulating antigen-specific memory B cells and/or plasmablasts from which the mAbs are isolated. In the case of HIV-1, suitable donors have been identified by screening large volumes of sera for their ability to neutralize viral isolates of multiple subtypes.[8–12] This process has resulted in the isolation of a large number of highly potent,

broadly neutralizing antibodies to HIV-1.[13,14] A similar approach has been applied to the isolation of a mAb that cross-reacts with RSV and metapneumovirus. In this example healthy donors with presumed past infection, by one or both of these viruses, were screened for serum activity against both viruses.[15] Broadly neutralizing influenza mAbs have also been isolated and have largely come from studies of pandemic survivors,[16–18] experimental and licensed vaccine recipients[19–21] and experimentally infected volunteers.[20] Since antigen-specific antibodies persist in the circulation for many years,[22] donor screenings can be performed long after infection, as was the case among 1918 Spanish influenza pandemic survivors.[18]

The characterization of antibody specificities responsible for serum neutralizing activity greatly facilitates efforts to isolate mAbs of interest. However, mapping antibody specificity is confounded by the fact that neutralizing antibodies are a minor component of the polyclonal antibody response. Nevertheless, multiple techniques have been developed for this purpose and used successfully. Peptide arrays, for example, screen antibody specificities through the presentation of overlapping peptides. Although they have been used for a number of infections, the general approach is limited by the fact that most broadly neutralizing antibodies recognize conformational epitopes and glycans that are not represented in the arrays.[23] The use of epitope-ablating mutants has also been helpful in mapping neutralizing antibody specificities, particularly those that target glycans on the HIV-1 envelope glycoprotein.[24–26] Depletion of plasma neutralizing activity through protein or peptide adsorption provides additional information for the design of soluble antigens that can bait antibodies of interest.[27,28]

The availability of large neutralization datasets has aided in the design of bioinformatic algorithms to predict specificities of serum samples, particularly in the case of HIV-1.[29–31] However, both experimental and computational epitope mapping methods are hampered by the presence of antibodies against multiple or undefined targets; in these cases mAb isolation may be necessary. While many of these technologies have been developed for the study of HIV-1, they have not been limited to this pathogen but applied others, like dengue virus, with great success.[32]

ISOLATION OF MONOCLONAL ANTIBODIES USING B CELL CULTURE TECHNOLOGIES

The first generalizable technique for isolating monoclonal antibodies was reported in 1975 by Kohler and Milstein.[33] This Nobel-prize winning pair fused an immortalized myeloma cell line (P3-X63Ag8) with mouse splenocytes to generate so-called hybridomas: stable cell-lines that secrete antibodies in culture. This process has led to the development of many mAbs that are still in use as immunologic reagents. Although the method represented a significant technological advance at the time, it carries a number of limitations.

Hybridoma technology depends on a high frequency of B cells that make the antibodies of interest. It has a generally low efficiency of hybridization between immortalized myeloma cells and splenocytes, and extension of the process from murine to human B cells has not been very successful.

Many large-quantity human mAb production protocols have been based on the transformation of B cells with Epstein-Barr virus (EBV), a human herpes virus with B cell tropism.[34] However, EBV entry into B cells is partially dependent upon CD21 expression,[35] which appears to be linked to in vitro B cell survival and proliferation. Over the past 20 years, investigations into B cell activation have found potent activators such as CD40L, IL-21, and CpG improve transformation efficiency either on their own or in concert with EBV. After activation, purified memory B cells can then be cultured over a short period (10−14 days), at which time supernatant is collected and screened for pathogen (usually virus) neutralization. High-throughput functional screening methods now make it easier to scan a large numbers of cultures in order to detect rare B cell populations[36,37] (Fig. 1.1A). As this technique is predicated on detecting functional antibody activity, donors must be selected carefully for their capacity to neutralize viruses at low antibody concentrations. Some of the most potent HIV-1 mAbs ever isolated have been found through this process, likely because of the stringency of the functional screening step.[37−39] A major advantage of using B cell culture to isolate mAbs is that the target epitope does not need to be predefined, thus enabling the isolation of antibodies that recognize new, previously unrecognized targets. Still, culturing memory B cells is relatively labor-intensive, requires large amounts of space and laboratory equipment, is prone to technical complications such as cell overgrowth and death and may result in antibody class switching during the culture period.[40] Multiple antibody lineages in one donor target the glycan-V3 supersite of the HIV-1 envelope glycoprotein and display a preference for quaternary binding.[40a]

ISOLATION OF mABs BY ANTIGEN-SPECIFIC SORTING

The identification of target epitopes by mapping antibody specificity allows for the design of highly specific antigens to sort out single B cells (Fig. 1.1B). One of the earliest and most successful applications of this technology involved the use of the resurfaced core 3 (RSC3) antigen of the HIV-1 envelope to isolate VRC01, a broad and potent CD4 binding site (CD4bs) antibody.[41] Interestingly, the majority of mAbs that were isolated by these means use a restricted set of germline genes.[42]

A number of techniques for sorting antigen-specific B cells have since been developed, but all of them display antigens that must be bound by immunoglobulins fixed to the B cell surface. As each method has particular advantages and disadvantages (Table 1.1), selection of the most suitable method depends greatly on the antigenic target. The simplest approach uses linear synthetic peptides that have a sequence motif (e.g.,

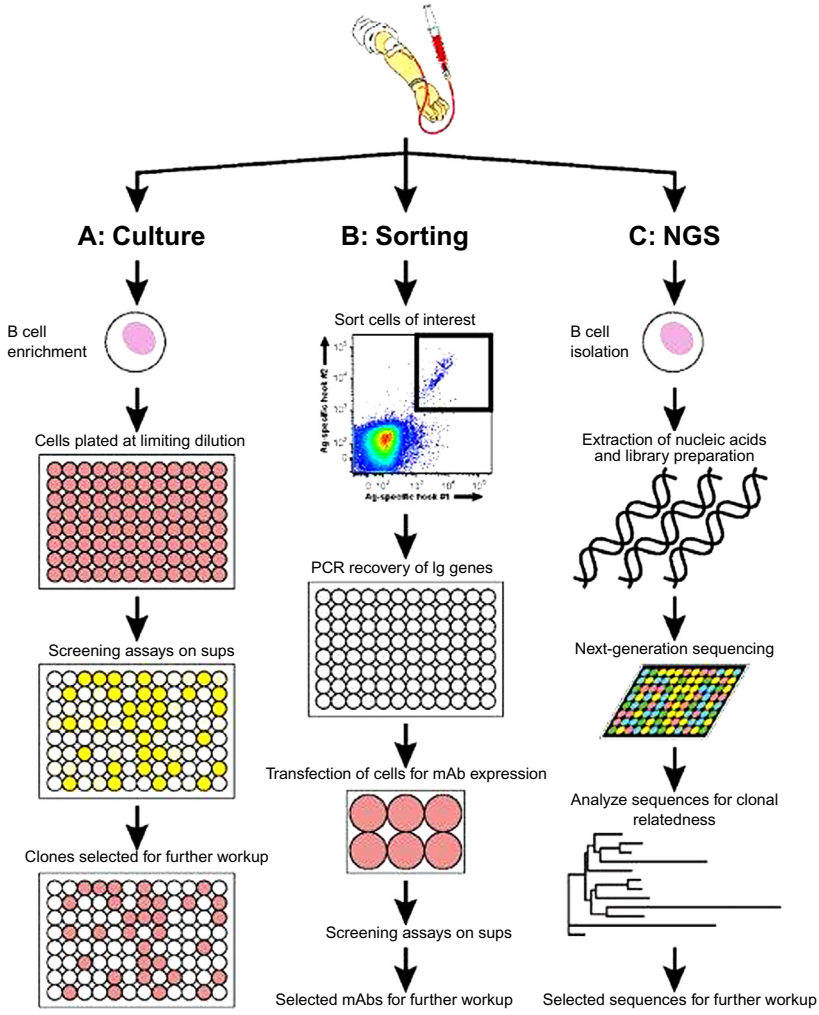

FIGURE 1.1 Schematic of the methods used to isolate monoclonal antibodies: (A) B cell culture, (B) B cell sorting, and (C) Paired reads of the immunoglobulin repertoire.

biotin) that can be designed in a manner that permits detection by a flow cytometer (e.g., fluorochrome-derivatized streptavidin). This technique was first used to identify a class of autoantibodies recognizing a mimetope of double stranded DNA[43] and subsequently used to isolate an antibody directed against the membrane proximal external region (MPER) of the HIV-1 envelope glycoprotein, gp41.[44] Peptide-based MPER reagents have also been used to characterize immune tolerance in mouse models,[45,46] work that has ultimately led to the identification of the autoantigen responsible for the

TABLE 1.1 Techniques for Isolation of Antigen-Specific B Cells

Technique	Advantages	Disadvantages
Linear peptides	• Simplicity • Ease of manufacture	• Depends on antibody recognition of linear sequence • Cannot be used for discontinuous or quaternary epitopes
Monomeric proteins	• Expression of epitopes in a more native conformation	• Requires protein synthesis • Cannot be used for quaternary epitopes
Multimeric proteins (e.g., trimers)	• Expression of epitopes in a more native conformation	• Requires the production of stable multimeric complexes
Virus-like particles	• Epitopes presented in situ on the surface of the particle, presumably in a native conformation	• Particle production can be difficult • Epitopes can be expressed in nonnative conformations • Can select for B cells reactive against other antigens on the particle
Cell-expressed antigens	• Epitopes presented in situ on the surface of the cell, presumably in a native conformation	• Requires production of cells expressing the antigen, can select for B cells reactive with other antigens on the cell • Relatively low specificity for isolated mAbs
Culture-based B cell recovery	• No need for prior knowledge of epitope targets • Screening can be based on functional assays (e.g., neutralization)	• Labor and resource intensive • Loss of cells of interest possible through contamination or outgrowth of irrelevant cells

regulation of this antibody class.[47] Although this technique is highly specific for epitopes presented as a linear peptide sequence, it comes at some cost. First, the method does not allow for the presentation of discontinuous or structurally complex epitopes. Second, it is poorly suited for in-depth characterization of antibodies generated in response to vaccination or infection.

Recombinant proteins are increasingly being used as baits to isolate antigen-specific B cells. For example, the highly specific CD4bs antibodies were isolated through a differential sort where the second screening antigen lacked the epitope of interest (e.g., RSC3 proteins with mutations at position