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Antibody Phage Display

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1. Introduction

Antibodies now constitute an indispensable tool for research across all areas of biomedicine and the life sciences. Whilst polyclonal antibodies from the serum of an immunized animal remain in widespread use, there is little doubt that monoclonal reagents have significant benefits. Foremost is their recognition of just a single feature (epitope) of the biomolecule of interest (the target or antigen). The conformation of the epitope as recognized by an antibody is of more than academic interest. Linear epitopes—for example, a contiguous stretch of amino acids on a protein antigen—are likely to be bound successfully by an antibody providing they are accessible. These features can be perturbed by chemical modification, but they are more likely to be recognized by the antibody after sample preparation (e.g., denaturation in the preparation of samples for a Western blot, or tissue sample for immunohistochemical analysis) than epitopes that are formed through folding of the target (e.g., conformational epitopes). These considerations may govern whether an antibody will recognize its target in the intended area of application.

1.1. Monoclonal Antibodies from Hybridomas

For around 30 yr, the hybridoma methods of Kohler and Milstein ***(1)*** have served as a general method for the production of monoclonal antibodies. The methods are based upon the immortalization of single B lymphocytes taken from a donor and immortalization by fusion with a myeloma cell line. When working with laboratory animals, the B-cell donor must be hyperimmunized with the antigen of interest to ensure that a high proportion of splenic lymphocytes are synthesizing antibody against the intended target. At least in principle, B cells specific for the antigen could be selected before fusion, but in practice this is rarely done. After cell fusion, selection for hybridomas is carried out with drugs that block nucleotide synthesis and salvage; surviving lines are cloned and screened for the synthesis of antibody against the antigen of interest. The system works well but is it labor-intensive and it is difficult to control the precise specificity of antibodies that emerge from screening. More fundamental limitations also exist. Because this method of monoclonal

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antibody production is founded upon immunization of a donor animal, problems arise in generating antibodies against self antigens (e.g., cell surface markers) or proteins of conserved sequence (e.g., proteins that a near identical between mice and humans). Molecules that are poorly immunogenic because of their structure (e.g., carbohydrates) are similarly problematic, and when mixtures of antigens are used for immunization, the host response is naturally biased to those antigens that are present in highest concentration or are most immunogenic to the host immune system. The method has obvious limitations when toxic molecules are the focus of interest. In a project with periodic or unpredictable requirements for monoclonal antibodies, immunization, fusion and screening must be carried out each time a new reagent is required. This has obvious cost and time implications.

1.2. Antigen-Binding Fragments

Over the past 15 yr, technologies have been developed that open up completely different avenues for the production of monoclonal antibodies. These methods are founded upon several conceptually simple principles but throughout, are recombinant in their philosophy. This route to monoclonal antibody isolation does not employ full-length antibodies as would be produced by the mammalian immune system – these can be constructed at a later stage if required – but instead works with antigen-binding fragments. Typically, these are Fab fragments or single-chain Fv fragments (scFv) (**Fig. 34.1**). The modest size of these proteins (55 kDa (Fab) and 30 kDa (scFv) versus 150 kDa for a full-length

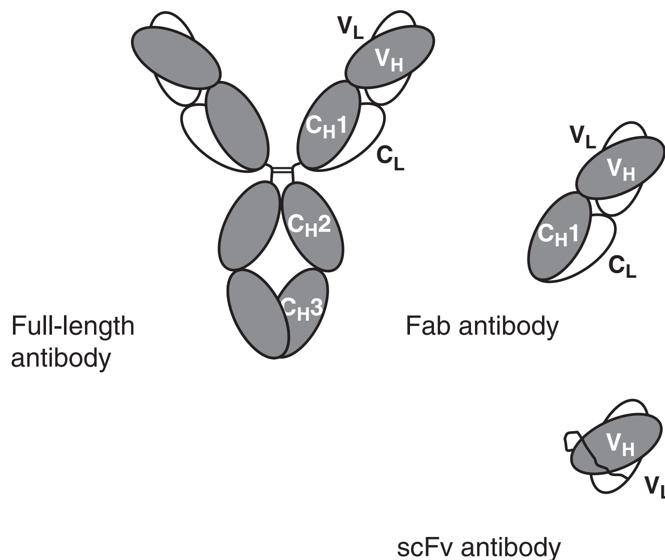

Fig. 34.1. Structure of full-length antibodies and antigen-binding fragments. Domains in the heavy (shaded) and light chain (open) components of these proteins are indicated. In the full-length structure, lines between the C_H1 and C_H2 domains indicate the hinge region of the immunoglobulin. The double connecting line in this region indicates disulphide bonds. In the scFv structure, the free-form line indicates a linker peptide extending from the carboxyl terminus of the V_H domain to the amino terminus of the V_L component

antibody such as IgG) makes possible their routine expression in bacteria. It is important to note however that these fragments retain the ability to interact with antigen, potentially with high affinity.

Fab fragments are conventially produced by proteolytic digestion of full-length antibodies using papain. Cleavage of the antibody in the flexible hinge region releases a monovalent antigen-binding fragment comprising the immunoglobulin light chain and the V_H and C_H1 domains of the heavy chain (**Fig. 34.1**). Fabs thus retain the original immunoglobulin's (Ig) capacity to recognize antigen and the constituent chains are held together by the natural forces of association.

In contrast, there is no natural equivalent of the scFv protein. The interactions between the V_H and V_L domains impart some stability but tethering through a flexible linker enhances this considerably ***(2,3)***. Translation as a single protein also enables the construction of simple expression systems for synthesis in bacteria. The construction of scFvs from antibodies often retains high affinity for the original antigen.

1.3. Aims of This Chapter

The objectives of this chapter are to explain how these antibody fragments can be constructed through molecular biology and how proteins that possess the ability to bind a biomolecule of interest can be conveniently isolated. It is significant that these goals can be accomplished quicker and with greater control than by application of hybridoma methods. The technology is also less prone to the constraints indicated above because the selection process takes place in vitro.

2. Methods

2.1. Overview of Antibody Phage Display

The generation of monoclonal, antigen-specific Fabs and scFvs by recombinant methods bears very little relation to conventional hybridoma technology. At the outset, a large library of antigen-binding fragments must be generated. Whilst the material for library construction can be sourced from the lymphoid tissues of animals or human subjects, entirely synthetic libraries have become popular ***(4–6)*** and have many advantages that are discussed below. Whatever its origin, the library is formed in a bacteriophage system so that each virus within the collection possesses at its surface an antibody fragment with the potential to bind to antigen, and carries within its capsid the coding sequence for that Ig ***(7,8)*** (**Fig. 34.2**). This linkage of phenotype (the ability to interact with a target molecule) and genotype (the coding sequence for the antigen-binding protein) is crucial for what follows.

Numerous options exist to extract clones from these phage libraries that have the ability to interact with a target molecule. Note that as libraries can be generated from nonimmunized biological sources or through synthetic means, the term "antigen" can become meaningless and "target" describes better the molecule against which antibodies are to be sought. In its simplest form, selection involves coating a plastic surface with the target molecule, and then application of the viral library ***(8)***. Those phage that display antibody fragments

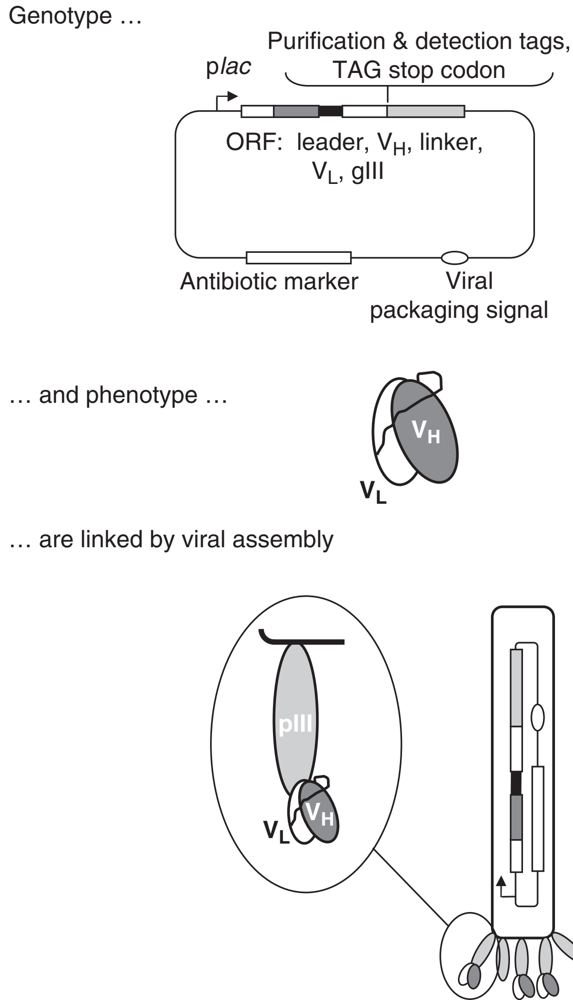

Fig. 34.2. The basis to scFv phage display. The components of a typical scFv phage display vector are shown at the top of the Figure with the organization of the soluble recombinant antibody product shown beneath. The domains of the scFv are labeled, the free-form line indicating the linker peptide extending from the carboxyl terminus of the V_H domain to the amino terminus of the V_L component. The foot of the Figure shows the schematic structure of a phage particle carrying the phagemid vector within the viral capsid (heavy line). The phage particle carries five copies of pIII (light shading) some of which are scFv-pIII fusion proteins (magnified section)

with affinity for the coated surface become immobilized on the surface; others can be washed away. Recoveries are typically low at this stage of selection so the phage are recovered from the surface, infected into bacteria and thereby replicated to much higher numbers. Through repeated rounds of selection, recovery and amplification, target-binding clones are recovered from the library and enriched for those that are favored by the conditions of selection.

At this stage, the antibody fragments are present at the surface of the virus, expressed as fusions to one or other of the components of the viral capsid. This

is essential if the antibody is to be displayed at the phage surface. Through simple manipulation, antibody fragment can also be expressed as an independent, freely soluble protein. Guided by a leader peptide (**Fig. 34.2**), the protein is then exported and released into the bacterial periplasm where folding – assembly in the case of Fab fragments – takes place ***(9,10)***. Many vectors encode peptide tags to enable purification (e.g., histidine repeats) and detection (e.g., c-myc, Flag) of the translation product (**Fig. 34.2**).

The characterization of viral clones recovered by phage display can take a number of paths. Firstly, immunoassay with either virus ("phage ELISA") or the individual protein ("soluble protein ELISA") can confirm the specificity of an antibody fragment for the target. The natural extension of these experiments is into Western blotting, epitope mapping and the determination of affinity through surface plasmon resonance. By sequencing the reading frame, the antibody fragment can be further characterized to determine the diversity of clones recovered. Purification of soluble antibody enables the biological properties of the protein to be assessed (e.g., seeking the ability of the antibody fragment to inhibit the action of the target molecule or processes in which it is involved). Adaptation of the antibody fragment can take many forms. The natural biological activity of a full length antibody can be restored by recloning the coding sequences into a mammalian expression vector that carries the antibody constant domains ***(11,12)***. The capacity of the fragment to bind to target can be harnessed by creating fusions with reporters (e.g., to locate the target molecule in cells ***(13))***, enzymes (e.g., for immunoassays for the target molecule ***(14)***) or bioactive agents (e.g., toxin fusions enable specific elimination of defined populations of cells in vitro or in vivo ***(15)***). With such a range of options, it is little wonder that phage display and recombinant antibody technology has rapidly become a popular experimental technique.

2.2. Library Construction

The flexibility of phage display as a method for the generation of monoclonal reagents becomes apparent even at the earliest stage of a project. Because of this flexibility, it is important for the investigator to be clear about experimental goals and the wider context of the project so that the most appropriate options are exercised. For many projects, a monoclonal reagent is simply required for detection of a biomolecule or for assessment of the function of this target in vitro or in vivo. If this is the case and the format (Fab or scFv) of the monoclonal reagent is not of great importance, then it many be possible to acquire a library from external sources and the time and costs of library construction may be avoided.

If the aim is to use phage display to learn more about the nature of the immune response in a patient or high affinity antibodies are required that have been matured by the response in vivo, the necessity for library generation from that patient, or from a hyperimmunized experimental animal becomes essential.

2.2.1. Source of Material

The construction of a custom library takes as its starting point, cDNA from an appropriate biological source that contains a diverse range of Ig transcripts. Many libraries have been prepared from human donors vaccinated against or

infected with particular pathogens or their products. For example, Zwick and colleagues have described the isolation ***(16)*** and characterization ***(17)*** of HIV-neutralizing antibodies derived from an HIV seropositive donor. Equally, the immune response active in patients with a range of syndromes and conditions can be sampled if access to lymphoid tissue can be arranged. Fostieri et al. ***(18)*** reported the isolation from myasthenia gravis patients of Fab antibodies the acetylcholine receptor. Note that samples need to be protected against the degradation of RNA on recovery and during storage so snap-freezing and storage at −80°C, and the use of proprietary protectants (e.g., RNAlater, Ambion) are strongly recommended. Samples from the spleen or lymph nodes are ideal for library construction, but may be hard to obtain from human sources. There may, for example, be ethical, cultural, or legal constraints on access to material from post mortem analysis, or tissues taken during surgery. Informed consent for the sampling of human blood may be easier and there are several reports in the literature of library construction with material sampled in this way ***(19–22)***. Before embarking on this approach, it is worth considering that the numbers of B lymphoblasts circulating in the blood are relatively low and the appearance of antigen-specific B cells may be transitory after infection, vaccination etc. These factors can constrain the yield of cDNA and the diversity of Ig transcripts for library construction. It is possible to isolate peripheral blood lymphocytes against a purified antigen ***(23,24)*** or to expand in vitro small numbers (even single) of B lymphocytes ***(25)*** to overcome the first of these issues.

To construct large libraries containing antibodies against many, chemically diverse targets, several investigators have deliberately chosen to sample the naïve human repertoire or to use material from normal donors. B lymphocytes bearing IgM at the surface can be selected from blood or lymphoid tissue. Alternatively, transcripts encoding IgM can be specifically recovered by PCR at a later stage driving the construction of the library towards the naive repertoire ***(26–29)***.

Sampling material from a vaccinee or an individual who has been exposed to a pathogen ***(30–33)*** can enrich a display library with high affinity antibodies against targets that may be of immediate relevance (e.g., toxins or other microbial virulence factors ***(34,35)***). The library may also represent a snap-shot of the diversity of the humoral response at the time of sampling, aiding analysis of the diversity of the response and its genetic basis (e.g., use of particular families of immunoglobulin genes ***(23,36)***). But these features may not be universally beneficial. The library may be overpopulated with clones that are reactive with immunodominant products recognized by the donor's immune system. The library will also be formed from all immunoglobulins expressed by the donor at the time of sampling, but not all antibody sequences express well in the bacterial systems used for propagation of the library, creating gaps in the repertoire of the library ***(37)***.

These sorts of limitations have been overcome with the generation of libraries that have been diversified by synthetic methods. Here, scaffolding sequences can be chosen that are known to be expressed successfully in bacteria and then diversified to produce libraries that can be screened for reactivity with a tremendous range of chemically diverse targets ***(38–41)***. This is the concept of a "single pot library" – a single resource that contains antibodies to practically any target ***(42)***.

2.2.2. Repertoire Recovery

Given a source of cDNA from a lymphoid tissue, the next objective is to recover from it antibody-coding sequences. The use of the polymerase chain reaction with specific primers allows extraction and amplification of antibody-coding sequences from all the other transcripts present in the sample.

Herein lies a paradox: of their nature, antibodies are translated from sequences of enormous diversity. How then can the repertoire be recovered with a manageable number of PCR primers? Fortunately, sequence diversity is concentrated into regions that code for the complementarity-determining regions (CDRs) – those parts of the protein that will contact antigen. The CDRs are supported on sequences (framework regions; FRs) that are more conserved in sequence. In the human and murine immune systems, antibody coding sequences are assembled from gene segments in the developing B lymphocyte that are numerous but which can be grouped into smaller numbers of families because of the conservation of the FR sequences ***(43)***. In many other species, the antibody repertoires are founded upon more limited use of Ig gene segments or families ***(44)***. Overall, the impact of these features is that huge molecular diversity can be recovered in a representative fashion through a significant but very manageable number of amplification reactions.

A major decision point arises at this point. Is the library to be constructed with Fab or scFv antibody fragments? This governs the actual primer sets that will be used for recovery of the repertoire. If Fab fragments are to be generated, a further question arises: is there to be a preference for antibodies of a particular class? With these considerations in mind, the human antibody heavy chain repertoire can be recovered by PCR with combinations of 8 primers that anneal to the coding sequence for FR1 and 4 that anneal to the coding sequence for IgG constant domain 1. Similar considerations govern the number of reactions and primer sets required for recovery of the light chain repertoires of mice and men (e.g., the de Haard library ***(28)***) although for other species, fewer primer sets are often needed because of more restricted usage of germline segments.

Near identical methods are used if it is decided that a scFv library is to be constructed, the exception being the nature of the primers that are used for amplification: primers specific for the FR1-coding sequence of light and heavy chain cDNA are paired with primers that anneal to FR4-coding sequence (e.g., the Vaughan library ***(27)***). The J segments that are rearranged in B cells to form this part of the Ig reading frame are modest in number in many species – in some animals, only single J segments are utilized – so once again, the repertoire can be recovered with reasonable numbers of PCR reactions using all combinations of heavy chain and light chain primers.

Depending upon the vector to be used for library construction, restriction sites are included in the primers used for PCR. In many cases, the combination of vector-derived sequence and the choice of the restriction site ensures near-native protein sequence in the recombinant product.

2.2.3. Library Construction

In the construction of large libraries from patient material, some investigators have chosen to clone heavy or light chain PCR products into a “holding” vector (e.g., the de Haard library ***(28)***). The purpose of this is 2-fold. First if materials are to be accumulated over a period of time (e.g., from patient material that for logistic reasons cannot be obtained regularly), it enables PCR products

to be archived in a stable form pending library construction. Second, when PCR products are digested for transfer into the display vector, thereby generating the library, excision of the inserts can be convincingly demonstrated and there is no ambiguity about their suitability for ligation. Restriction enzymes chosen for cloning are usually able to digest DNA close to the termini of the duplex and where efficiency is an issue of concern, recognition sites can be positioned to ensure efficient digestion. However, excision of the amplicons from a holding vector provides clear confirmation that the antibody sequences are ready for ligation.

Phage display vectors have been extensively adapted by different investigators to match individual needs, but the following properties are typical, irrespective of whether scFv or Fab libraries are to be prepared (**Fig. 34.2**). For display upon filamentous phage, pIII is the preferred anchor ***(7)*** and phagemid vectors have superseded the use of viral genomic DNA except for specialist purposes (e.g., multivalent display ***(45)***). This is largely because shifting from single-stranded DNA carried within the viral capsid to an independently replicating, double-stranded plasmid in bacteria (ideal for purification in high yield, restriction analysis, sequencing, and protein expression studies) is extremely straightforward. Aside from a plasmid origin of replication, a selectable marker and viral sequences to enable packaging into virions, these vectors possess a promoter for expression of the cloned antibody sequence (typically p*lac* for ease of regulation), bacterial leader sequences (e.g., the *pelB* and *ompA* leader sequences) and cloning sites for the Ig inserts (**Fig. 34.2**). Fab display vectors usually carry a simple dicistronic operon for independent expression, translation and export of the heavy and light chain components (e.g., pComb3; ***(7)***). In scFv display vectors, the linker sequence that tethers the V_H and V_L components can be included in the vector, or introduced by PCR before ligation into the vector. In order that the antibody fragment will be displayed, the Ig sequence lies in-frame with gIII, the coding sequence for pIII, a minor phage coat protein present in five copies at the tip of the assembled virus (**Fig. 34.2**). The pIII protein mediates infection of the bacterial host by the phage through interaction with the bacterial F pilus and the TolA co-receptor ***(46)***. Pioneering work by Smith ***(47)*** demonstrated that phage infectivity could be retained even when peptides and proteins were fused to pIII. A range of features may be present in the intervening sequence of the display vector. It has become conventional to include an amber stop codon in this part of the vector (**Fig. 34.2**) providing the option of expressing an antibody-pIII fusion protein for display in suppressor strains of *Escherichia coli* (e.g., TG1). Moving the construct into a nonsuppressing bacterial host (e.g., HB2151) then allows expression of the antibody fragment as a soluble protein. The same goal can be achieved with only slightly more effort by excision of the gIII sequence using flanking restriction sites that generate compatible termini, and religation ***(7)***. Histidine repeats to facilitate protein purification and tags for immunochemical detection are also common features of this part of the vector (**Fig. 34.2**).

Once the PCR products from repertoire recovery have been cloned into display vectors and transformed into an appropriate strain of *E. coli*, how then is viral assembly initiated? Only two viral sequences are present in the vector: gIII and the packaging signal. By adding helper phage (e.g., VCS M13, Stratagene), the full range of viral proteins can be synthesized in the bacterial host, viral assembly can begin, but it is a single-stranded form

of the phagemid rather than the helper phage genome that is packaged into progeny phage. Filamentous bacteriophage are not released from *E. coli* in a catastrophic lytic event (*cf* phage lambda or the T phages) – rather, they are extruded as viral proteins accumulate in the inner membrane and assemble to form the capsid. After DNA is packaged into the virion, the capsid is capped with copies of the minor coat protein pIII. This is a crucial stage as regards the formation of a display library. Low level expression of the antibody-pIII fusion from the phagemid is achieved by growth of the bacteria in low concentrations of glucose thereby allowing some expression from the *lac* promoter. This leads to accumulation of the recombinant protein in the bacterial membrane. As capping takes place, a mixture of pIII proteins – wild-type pIII encoded by the helper phage and antibody-pIII from the phagemid vector – are incorporated into the capsid (**Fig. 34.2**). It is estimated that one or two fusion proteins are most commonly incorporated ***(48)***, the balance comprising wild-type pIII. Thus progeny phage carry the phagemid, and display at their surface the encoded antibody fragment. When these events take place across the stock of bacteria transformed with the recovered Ig repertoire, a phage display library results.

The properties of helper phage used in this procedure have been extensively refined over recent years. Selection of the library on the target biomolecule can be aided by elimination of phage that only display wild-type pIII and interact with the selecting surface in a nonspecific fashion. Helper phage such as KM13 have been modified to include a trypsin-sensitive site in pIII ***(49)***. Treatment with the enzyme renders them noninfective. When partnered with a display vector that expresses pIII lacking this susceptibility, proteolysis can be used to inactivate any phage that lack antibody at their surface. An alternative approach is mutation of the helper phage such that it carries a truncated version of gIII that is unable to contribute to the infection process ***(50)***.

2.2.4. Characterization of the Library

Once the display library has been generated, its size and potential utility can be estimated in a number of ways. The absolute number of virus present can be determined by titration along the simple principle that infection of bacteria with phage will transduce the vector into the bacterial host thereby conferring upon the bacteria resistance to antibiotic. Samples of the library are thus serially diluted and by adding aliquots to cultures of bacteria that express the F pilus, the frequency with which bacteria are converted to antibiotic resistance can be established. Phage stocks of 10^{12} transducing units per ml or more are routinely obtained.

Plasmid DNA can be re-isolated from bacteria infected in this way for further analysis. Restriction analysis of colonies picked at random can determine the frequency of complete Ig inserts within the library. The ligation of amplicons into the display vector is rarely 100% efficient ***(48)*** and it is useful to know what proportion of the library lacks the heavy or light chain components of a Fab or the V_H or V_L regions of a scFv.

The diversity of Ig sequences carried by the library is also an important parameter. This can be estimated by digesting the Ig inserts with restriction enzymes like *Bst*NI, an enzyme that recognizes a 4 base sequence (5′ CC(A/T)GG 3′) that happens to occur commonly in Ig V region coding sequences. Given the likely occurrence of this sequence in the display vector, it is important that the antibody V region insert is specifically recovered by PCR or restriction digestion

before analysis. Failing this, the restriction profile is likely to be obscured by vector-derived fragments.

A more detailed assessment of library diversity can be made by sequencing. For scFv inserts, sequencing primers can be designed against upstream or downstream flanking vector sequences or either strand of the linker region. For Fab inserts, the V region sequences are rather shorter and hence it may be possible to sequence the region of interest using primers against the bacterial leaders upstream of the V_H and V_L inserts. Although more costly and time-consuming, this approach has several significant advantages over restriction analysis. The extent and location of diversity can be thoroughly assessed. If some amplicons from repertoire recovery are disproportionately represented in the library, this can be identified. The degree of diversity present in the library can also be assessed. Perhaps the most important benefit of sequencing is that the reading frames in the library can be checked for integrity. cDNA prepared from lymphoid tissue will include transcripts from B cells undergoing somatic hypermutation, a process with the capacity to introduce stop codons and (more rarely) shifts in the reading frame. Similarly, the use of PCR for repertoire recovery will introduce errors with low frequency. For synthetically diversified libraries, stop codons will be introduced a higher frequency. An alternative method that can be employed to check for the integrity of the reading frame is to pick clones at random from the library and after appropriate manipulation (*see* the following), check for the expression of soluble, recombinant antibody by capture ELISA, dot blotting or Western blotting.

2.3. Screening by Phage Display

2.3.1. Selection Methods

One of the most attractive features of phage display is the speed with which selection from the library can be executed, the flexibility of the method of selection and, most of all, its potential for direct extraction of antibodies directed against the target of interest. It is worth comparing this latter feature with conventional monoclonal methods where the only driving force towards the antibody of interest is the frequency of B cells of the desired specificity amongst the hybridoma population.

In its simplest form, selection can take place upon a convenient surface (often plastic) that has been coated with the target ***(8)***. This is an application of the “panning” methods originally devised by Smith ***(51)***. As in most immunochemical procedures, the surface needs to be blocked to minimize nonspecific binding of library phage. To avoid capturing phage that bind to the blocking reagent, the blocker (often skimmed milk, but purified proteins like serum albumin or gelatine are also used) is preincubated with the phage stock. This reduces substantially the chances of interaction with blocking protein present at the selecting surface. The number of phage added to the surface usually ensures that each specificity calculated to exist in the library is represented several thousand-fold, allowing ample opportunity for recovery. After incubation, the surface is washed rigorously and those phage that remain attached are recovered. Recovery can be though change in the pH ***(48)***, the addition of mild chaotropic agents ***(5)*** or proteases for those phage systems that are appropriately adapted (*see* description of KM13 above ***(49)***). Whilst elution must break the interaction between the displayed antibody and the target-coated surface,

it must not be so harsh as to impair the ability of virus to infect bacteria. Fortunately, the infection process is robust.

Recovered phage are then infected into an appropriate strain of bacteria. This serves two important purposes. Firstly, it allows an estimate to be made of the number of virus recovered from selection; as before, this can be done by measuring the frequency with which bacteria are transduced to antibiotic resistance. Secondly, the numbers of phage recovered at the first round of selection are modest (numbers in the range of 10^3 to 10^4 are typical) and represent a very low percentage of the input (10^{10} to 10^{12}). Infection into bacteria, recovery of antibiotic-resistant colonies and reinfection with helper phage allows amplification of the recovered sample to numbers appropriate for a second round of selection. Further rounds often take place on surfaces coated with successively lower concentrations of the target molecule in an effort to refine selection towards those antibodies with the highest affinity of interaction ***(48)***. The percentage of phage recovered at each round of selection often climbs sharply from round one to two and further elevation may occur at round 3 ***(8,51)***. This can be suggestive of success. Sequencing of clones picked at random through a selection experiment often reveals the emergence of common sequences in the CDRs from collections that initially appear diverse. This indicates that progressive enrichment is taking place during phage selection. It is important to note, however, that this selection is "blind" and that despite careful experimental design, it can drift from the intended direction for a number of reasons. For example, phage that have a low propensity to interact with the selecting surface but are able to replicate quickly will rapidly dominate the output. Similarly, antibodies that react with a contaminant with high affinity rather than the intended target can emerge. Selections that suffer these problems can show all the signs of success as selection proceeds but their failure will only become apparent when tested in immunoassay at a later stage. If these problems arise, again the speed of phage display means that setbacks can be quickly rectified.

The formats for selection are limited only by the ingenuity of the investigators. To avoid conformational change in the target by binding to a plastic surface, solution-based selection methods are popular. Often the target is modified by addition of a small ligand (e.g., biotin). Phage mixed in solution with the target can then be captured to a plastic surface coated with streptavidin or collected by using magnetic beads ***(52,53)***. Selection in solution can also be followed by capture to a surface coated with another antibody directed against the target. This selection method is also useful for small target molecules that may not bind efficiently to plastic or when direct binding to a selecting surface may obscure features of potential importance.

Antibodies against small target molecules can also be isolated by phage display through the use of carrier proteins. The target can be coupled to a larger protein carrier either by chemical activation or through genetic fusion and the conjugate then coated to a selecting surface. Naturally, antibodies may be bound during selection via interaction with the carrier. To drive selection towards the molecule of interest, selection in the next round takes place using conjugate employing a different carrier protein ***(54)***.

This leads an important principle that can be employed in phage display – the concept of negative selection. Although selection strategies naturally spring to mind in which clones that bind to a coated surface are retained and

carried forward to successive rounds, it is equally possible to deplete on a coated surface, leaving behind those antibodies that are captured. This is valuable when seeking antibodies against some feature of the target, one that distinguishes it from other, closely related biomolecules. By applying the library to an irrelevant but similar target, the library can be depleted of antibodies directed towards common or shared motifs (negative selection step). Phage left free in solution can then be taken forward for positive selection against the target. Alternating rounds of negative and positive selection may then drive the process towards the specificities of interest ***(55)***.

These examples only serve to illustrate some aspects of selection. Recent reviews ***(4,5)*** should be consulted to appreciate the enormous variety of selection strategies that have been employed by investigators.

One common feature of selection alluded to earlier is that round-on-round, the diversity of the recovered clones falls as enrichment takes place. When using complex targets or those comprising multiple molecular species, antibodies of potential value are discarded during early rounds of selection. To overcome this wastage, robotic methods have emerged. These are of particular value when deriving antibodies for use in proteomics. Thousands of clones are picked for evaluation at the early stages of selection, and checked by gridding onto membranes coated with the targets of interest ***(56,57)***.

2.3.2. Assessing the Output

The assessment of phage numbers can be conveniently determined by titration experiments. This is important in calculating the size of a library, recoveries of virus during selection and amplification when preparing for later rounds of panning. The procedure exploits the ability of phage to infect strains of *E. coli* and transduce them to express the antibiotic marker carried by the phagemid. Hence, serial dilution of the phage stock and infection to *E. coli* TG1 provides a convenient assay.

When selection successfully extracts clones from a library, it is customary to observe low percentage recoveries at the first round of selection that rise by several orders of magnitude in the next round. Further elevation may be seen in later rounds of the selection. This does not necessarily imply that clones against the intended target are emerging but it is a strong indicator of selection on some basis.

The specificity of clones recovered from selection can be conveniently determined by ELISA using various formats. One assay of immediate benefit takes the mixed output of phage from a round of selection and tests its reactivity against the target and a range of irrelevant proteins or other biomolecules. Binding of virus to the immunoassay surface can be detected with reagents against the capsid. Depending upon the supplier, the reagent may be directly conjugated to an enzyme reporter (e.g., horseradish peroxidase) or addition of a secondary antibody–enzyme conjugate may be required. Because the viral input to the assay is mixed in composition, the assay is often termed polyclonal phage ELISA. It can be usefully applied in an initial assessment of the success of a selection protocol. Along with the rising percentage recoveries of virus, increasing ELISA strength in the polyclonal phage ELISA versus the intended target provides signs of specific selection, assuming signals against an irrelevant target remain consistently low. The assay can, for example, confirm that viruses are not emerging through inadvertent selection against blocking materials used in panning.

The natural progression in evaluating the output from selection is to identify individual clones that react with the intended target. Plates used for titrating the output of each round of selection can be used to isolate individual clones for culture, superinfection with helper phage, and the preparation of monoclonal phage stocks. These can then be tested in ELISA as described above.

Some caution has to be exercised with the outcome of monoclonal phage ELISA. Because these assays are typically executed with many clones simultaneously – it is common practice to pick, for example, 96 clones from the output at each round of selection, using microtitre plates for bacterial culture and superinfection – it is rarely possible to titrate the numbers of virus used in the monoclonal phage ELISA. Hence, fluctuations in the ELISA data among clones under test may reflect to some extent variation in the viral input rather than strength of interaction with the target. Another *caveat* is that phage-based ELISA can generate signals of apparent strength but if the affinity of the displayed antibody for the target is moderate or low, the signal may be heavily dependent upon multivalent display (i.e. it benefits from avidity effects rather than affinity). In this instance, expression of the antibody as a monovalent soluble protein fails to generate the signal strength in ELISA that might be expected from initial testing with phage. The final aspect of the technology that needs to be considered is the influence of the bacterial host. Propagation of virus is often carried out in suppressor strains of *E. coli* (e.g., TG1) to ensure translation of the amber stop codon that (depending upon the vector) may be positioned between the antibody and pIII reading frames. This is essential for synthesis of the fusion protein required for display. In this host background, amber stop codons elsewhere in the reading frame (e.g., located in the CDRs where diversification may have been generated synthetically) will also be translated ***(56)***. Phage carrying these sequences may be able to bind to the target biomolecule in ELISA. When the construct is transferred to a nonsuppressing bacterial host (e.g., HB2151), a full-length antibody cannot be formed so once more, clones that appear promising from monoclonal phage ELISA are unreactive at a later stage of their characterization. For all these reasons, it is vital that a significant number of clones are chosen from monoclonal phage ELISA for further characterization, and that this choice does not entirely favor clones that generate the strongest signal in phage ELISA.

These issues concern the viral input to ELISA. It is also worth considering the nature of the target at this point in the evaluation process. The outcome of the assay will be used to judge if phage recovered from the screen are of the intended specificity: it is therefore vital that the target used for ELISA is as pure as possible or controls are included in the assay to determine if phage have been isolated that are reactive with a (potentially minor) contaminant, the blocking agent used in selection or another component of the selection system (e.g., naked plastic, biotin, streptavidin etc.). On the assumption that clones recovered from selection are of the intended specificity, it may also be possible to assess at this stage if recognition is taking place of a particular feature of the target biomolecule. For example, competitive ELISA with another monoclonal antibody, a ligand or other biomolecule reactive with the target, or peptides derived from the target sequence can be used to good effect. Finally, the coating of the immunoassay surface with different forms of the target (e.g., the native protein if a recombinant form has been used in selection, close homologues of the target perhaps taken from other species, protein that has

been modified at a post-translational stage [phosphorylation, glycosylation, addition of fatty acyl moieties]) or chemical derivatives of small target species (e.g., forms of the target that lack particular molecular features, alternative peptide sequences) can help define antibodies that show particular promise at an early stage of their characterization.

Clones that emerge from this phase of analysis can be sequenced to assess the diversity of the CDRs or (for libraries derived from an immunized source) the range of Ig segments that contribute to the response against the target in vivo. Consensus sequences may appear in the CDRs of antibodies isolated by stringent selection methods, but this step is also worthwhile in identifying whether identical clones are present. Because the DNA of phagemid display vectors is easily isolated for sequencing, this approach has significant benefits over assessing the diversity of recovered clones by restriction analysis with *Bst*NI or other frequently cutting endonucleases. As described above, primers for sequencing may be designed against flanking regions of the antibody reading frame or the coding sequence for the scFv linker.

2.4. Expression of Recombinant Antibodies

Most display vectors have been designed to minimize the degree of manipulation in moving from expression of antibody-pIII fusions to synthesis of soluble, monovalent protein. For some (e.g., the Fab display vector pComb3 ***(10)*** and its derivatives), the pIII coding sequence is excised by digestion with *Spe*I and *Nhe*I. Because these enzymes generate compatible termini, the vector fragment is then isolated and re-ligated for expression of the Fab in an *E. coli* host. Many scFv display vectors possess an amber stop codon between the reading frames for antibody and the phage coat protein (**Fig. 34.2**). Infection of virus into a nonsuppressing host (e.g., *E. coli* HB2151) therefore leads to expression of the soluble antibody fragment.

In the majority of display vectors, transcription of the recombinant antibody takes place from a *lac* promoter. It is customary to grow the bacteria under glucose repression until adequate biomass has been reached. Removal of the glucose by centrifugation and resuspension in fresh, glucose-free medium containing IPTG inducer then triggers transcription and translation. The choice of growth temperature during the expression phase is important to avoid aggregation of the protein and to minimize toxicity to the bacterial host though some studies suggest that effects on yield can be minimal ***(58)***. Expression at 30°C is often chosen as a starting point. Frequently, overexpression of the antibody leads to leakage of the periplasmic contents to the culture medium from which the protein can be purified. Purification is aided significantly by the presence of a histidine repeat sequence at the carboxy-terminus of the protein, a feature that is most conveniently incorporated in the original vector. Material generated in this way can be used in immunoassay or blotting, detection being made with Ig-binding proteins such as Protein A, G or L ***(56)*** or reagents against the purification and/or other peptide tags (e.g., c-myc, Flag etc.).

Because analysis of the soluble protein differs in many ways from that of the original phage clone, it is prudent to carry forward many different constructs that appear to bind to the target. The movement from a suppressor mutant for expression of a scFv-pIII fusion and propagation of phage to a

nonsuppressor host for expression of soluble protein can mean that clones that are positive in phage ELISA react poorly when the soluble antibody is expressed (*see* above). Amber stop codons present in the Ig reading frame will truncate the antibody prematurely in this situation. Some Ig sequences can be expressed more successfully in bacteria than others and whilst some investigations have identified causes for this effect ***(59)***, in many cases, they remain unexplained. Clones from custom, immunized libraries may therefore prove difficult to overexpress if they happen to carry problematic framework sequences. It is for this reason that some of the most successful, synthetically diversified libraries are founded upon single frameworks: the framework can be chosen as one that expresses consistently well in a bacterial host ***(56)***. The affinity of the antibody-target interaction can also be a relevant consideration ***(60)***. Multiple display of an antibody at the phage surface can drive a promising interaction in phage ELISA but when expressed as monovalent soluble protein, constructs with low or modest affinity for the target can give ELISA signals that are close to background.

Some of these potential problems can be foreseen by careful experimental design. For example, Westerns, dot blots or capture ELISA can be used to check for evidence of expression of the protein before target-specific ELISA is undertaken. These data can also be used to normalize extracts or quantities of purified protein that are taken forward to ELISA so that reaction with target can be ranked in the knowledge that roughly equivalent amounts of recombinant antibody have been used in the assay.

Once these issues have been addressed, expression of the antibody can be scaled up and the product purified by nickel chelation chromatography (if a histidine tag is present), or affinity chromatography on Protein A, G, L (according the antibody sequence carried). Determination of the affinity of the purified protein for its target is most commonly assessed by surface plasmon resonance using instruments such as the BiaCore.

2.5. Optimization and Further Modification

Once recombinant antibodies have emerged from selection and characterization, the availability of the coding sequence and the ability to express the protein in bacteria opens up numerous options. The affinity of the antibody for its target can be enhanced by rational or random mutagenesis ***(15,61,62)***, manipulations that may further refine the specificity of the antibody for its target. Fusions can be generated, linking the Ig reading frame to enzymes like alkaline phosphatase ***(63)*** or fluorogens like green fluorescent protein ***(64)***. Other manipulations (e.g., the addition of a terminal cysteine residue ***(65)*** can aid the covalent attachment of other moieties ***(66)***. Coupling of the recombinant antibody to other protein domains of modest size (e.g., the human kappa constant domain ***(67)***) or other entities (e.g., maltose binding protein ***(68)***) may improve yields and / or enable more convenient detection of antibody binding.

Reconstruction of a full-length antibody from a scFv or Fab protein may confer biological activities such as the ability to activate complement upon target recognition but proteins of this size often require expression in eukaryotic systems. The availability of specialized vectors ***(11,12)*** can assist in achieving this goal.

3. Applications

3.1. Tools for Proteomics

Antibody libraries can be mined for reactivity against a wide range of targets and then to use the recombinant antibodies as reagents for target detection and characterization: this is well-illustrated in the area of proteomics. Ohara and colleagues compared the properties of recombinant Fabs from a library of synthetically diversified human antibodies with polyclonal antisera against the same targets and reported that the Fabs were well-suited to Western blotting and immunohistochemistry ***(69)***. Earlier, de Wildt et al. described experiments in which one or two rounds of conventional screening were used to isolate phage from synthetically diversified libraries constructed on single V_H and V_L frameworks. Clones (up to 12,000) were then picked and gridded out using robotics onto membranes coated with the targets to test their specificity ***(56)***. The study demonstrated the capacity of large display libraries to yield antibodies against a wide range of targets and the ability to extract antibodies against minor components of complex mixtures, given appropriate (robotic) methods for identification of the recognition of target. Other investigators have described phage selection using blots prepared from 2-dimensional gels – a use of the proteome itself as the target for extraction of specific antibodies from a library.

For naïve or synthetically diversified libraries, the diversity of the resource is such that extraction of antibodies against human protein targets (e.g., tumor necrosis factor ***(2)***, human chorionic gonadotropin ***(28)***), molecules that are highly conserved amongst eukaryotes and therefore poorly immunogenic (e.g., ubiquitin ***(56)***) or toxic compounds (e.g., doxorubicin ***(5)***) is possible.

Antibodies isolated through phage display can be developed as diagnostic reagents but a more intriguing prospect is their use in construction of microarrays upon which binding of multiple analytes could be detected ***(40)***. Phage display is particularly valuable in this area of application because a single, highly diversified resource (the library) can be conveniently mined for antibodies against very many different targets. Some authors have described the use of stringent selection methods to isolate antibodies of very high affinity *via* phage display ***(70)***. These reagents then have the ability to detect very low concentrations of an analyte. For example, Wang and colleagues have reported the formation of a bivalent scFv fusion to alkaline phosphatase that in immunoassay, could detect as few as several hundred *Bacillus anthracis* cells in 2 hours ***(71)***. They further showed that replacement of the enzyme reporter with Cy3 dye increased sensitivity of the assay by about 10-fold with no increase in the time taken to conduct the assay.

3.2. Antibody Therapy

When the target is a molecule linked with a disease state, there are numerous reports of the isolation by phage display of antibodies with therapeutic potential. Infectious diseases have proved a productive area for investigation and in one example, antibodies against anthrax toxin have been shown to neutralize the lethal properties of this bacterial virulence factor ***(72)***. The pathogenesis of established (e.g., rotavirus ***(73)***) and emergent viral agents (e.g., SARS ***(74,75)***) can also be blocked by recombinant antibodies. Antibodies generated

in this way have the potential for treatment of acute infection, blocking the disease process at a key point (e.g., interaction between a receptor and the pathogen ***(76)*** or its product(s) ***(72)***). This provides a therapeutic option when the use of antibiotics or antiviral compounds is undesirable or impossible ***(77)***. It also offers prophylaxis when vaccines are unavailable ***(78)*** or the patient is unable to mount a protective response as a consequence of vaccination ***(79)***.

These areas of application deal with infectious agents, but antibody phage display has also been widely applied to the search for antibodies with antitumor activities. Careful design of the selection strategy has enabled, for example, investigators to isolate antibodies that are specific for markers expressed on transformed cells but absent from nontransformed primary cells ***(80)***. Therapeutic application need not trigger complement – recruitment of effector cells ***(81,82)*** can be exploited and in some cases, internalization of the recombinant antibody can have directly antiproliferative effects ***(83)***.

3.3. Analysis of Intracellular Processes

Practically all conventional applications of antibody phage display use the recombinant proteins as free, soluble reagents. However in an important exception, intrabodies are deliberately retained in the cytosol ***(84)***. This is an important and growing area of application because it provides a natural complement to mutation, gene knockouts and RNAi in the analysis of gene function.

To create an intrabody, the coding sequence of an antibody of some chosen specificity is recloned into a mammalian expression vector – crucially, the insert lacks a leader sequence – and the construct is then transfected into cells. Through this manipulation, the translation product is confined to the cytosol of the transfected cell. If the antibody is successfully folded, it can bind to other molecules present in this location, potentially blocking their normal function. Tagging the antibody with targeting motifs can direct relocalization of the antibody to the nucleus, the mitochondria etc.

Intrabodies find immediate application in the analysis of viral gene function ***(85,86)*** where interactions between viral proteins and host factors can be disrupted through binding of recombinant antibodies in the cytosol. Other pathogenic processes – inherited conditions like Huntingdon's disease ***(87)***, Alzheimer's ***(88)*** and cancer ***(89)*** – can similarly be analysed through the application of intrabody techniques.

This approach enables the definition of pathways linked with pathogenesis or the contribution of defined proteins in normal cellular processes. Given the complications of delivering an antibody into the intracellular environment, it perhaps serves more as a route to target discovery and validation than a direct route to therapy.

3.4. Directing Drugs or Other Therapeutic Compounds

Lastly, the specificity of antibodies can be exploited for the specific delivery of drugs and other therapeutics. Obviously, the use of phage display can speed the isolation of antibodies with the desired targeting properties. Genetic fusion has been used to link recombinant antibodies to other proteins that possess toxic activity ***(15)*** but the introduction of nonnative residues at the terminus of the recombinant antibody has also allowed the application of conjugative chemistry ***(66,90)***. By linking enzymes to the antibody, nontoxic prodrugs can

be converted to their active form in situ for anticancer therapy ***(91)*** or thrombolytic agents can be delivered ***(92)***. Recombinant antibodies have also been used for the delivery of drugs across the blood brain barrier ***(93)***.

3.5. Future Directions

The flexibility of antibody phage display is such that applications are limited only by the imagination of the investigator. Aided by appreciation of the advantages of phage display methods over hybridoma technology, the literature has now expanded enormously. In consequence, this chapter can only provide an overview of how antibodies can be isolated using these methods, and a brief insight to their potential applications. It is inevitable that the use of these methods and their exploitation will continue, driven by the opportunities from "omics" biology and the need for new therapies.

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