Review

Bacteriophage-derived antibodies in cancer research – diagnosis, imaging, and treatment

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1. Introduction

The production of monoclonal antibodies (mAb) from B cell hybridomas was a major landmark in immunology, providing both scientific and clinical tools useful in the identification and treatment of various diseases. In the twenty-five years since the discovery by Köhler and Milstein many thousands of monoclonals have been tested but surprisingly few have reached the clinic for a variety of reasons (reviewed in [19]). One of the main problems in using murine mAb *in vivo* is their antigenicity, a problem circumvented in part through antibody engineering or the generation of human hybridomas.

An alternative approach that completely bypasses hybridoma technology is bacteriophage antibody display, where antibody fragments are expressed as fusion proteins on the surface of filamentous bacteriophage (or phage). This approach was first reported by McCafferty et al. [36] who expressed the variable heavy and light chain domains of the anti-lysozyme antibody D1.3 on phage, subsequently this technology has been applied to a plethora of target molecules from plants to humans by many different groups around the world.

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The construction of large diverse repertoires of antibody fragments, either single chain Fv (scFv) or Fab, from naïve or immunised donor B cells has now become relatively standard thanks in a large part to the work of Greg Winter and his group in Cambridge, UK. A number of excellent reviews already exist describing the preparation [53], selection strategies [26], and future potential of this approach [24]. Obviously phage-derived antibodies have benefited from their mAb heritage in terms of well-understood structure, screening assays, and availability of secondary detection reagents and therefore it is hoped that clinically useful reagents can be produced more quickly than their hybridoma ancestors. Here, we concentrate on the contribution that bacteriophage display antibodies have made to cancer research and discuss a number of reagents, which have been developed for use in tumour identification, imaging and immunotherapy. In addition we attempt to highlight the future potential of this technology for the synthesis of new screening and therapeutic species.

The choice of which type of library, naïve or immunised, depends mainly on the intended application. For instance, to isolate antibodies with novel specificities against a given molecule (particularly one which is not immunogenic) or to identify new antigens, such as tumour or disease-associated molecules, the use of a large unbiased library is favoured, because it provides the widest rage of antibody fragments. On the other hand, an immunised library that makes use of the donor's immune system is preferable when antibodies with a higher binding affinity for a given antigen are required [26], although it must be remembered that such libraries will possess a bias towards immunogenic targets. Additionally, the choice of the selection strategy has a critical effect on the outcome of the isolation process [1]. As antibody selection is based solely on reactivity with the target, in most cases the isolated phage antibodies will only bind tissue presented in a similar form, i.e. antibodies selected on frozen tissue

recognise fresh or frozen tissue, but not formalin-fixed paraffin-embedded tissue, and *vice versa*.

Within the field of cancer, purified or biotinylated antigens coated on immunotubes, cell lysates, whole cells, and tissue sections have all been used successfully for the isolation of various antibody fragments from the different kinds of libraries. We have chosen to review the field looking first at work with naïve libraries then move on to the immunised libraries, rather than follow all the work on a particular type of tumour or target molecule. This approach will allow us to assess the relative merits and drawbacks of each type of library more easily. Table 1 summarises all the clones that have been described in the literature to date.

2. Phage antibodies from non-immunised libraries

Non-immunised libraries can be either naïve or semi-synthetic (see Fig. 1). Naïve libraries are constructed from the light-chain and the heavy chain V-gene pools from non-immunised healthy individual [34]. High quality naïve libraries display a large panel of different antibodies specificities ($\geqslant 10^8$ clones [39]) and generally provide a wider range of specific antibodies than the immunised repertoire libraries (approximately 10^7 clones [1]). Semi-synthetic libraries are composed of cloned V genes in which one or more of the CDR regions of the heavy chain, in particular CDR3 that contains the greatest structural diversity, have been randomised and assembled *in vitro* using PCR or oligonucleotide-directed methods [2,14, 27]. The introduction of new sequences, which are not naturally occurring increases the library diversity. Nevertheless, randomising the CDR sequence can also lead to a reduction in the number of functional antibody fragments that are displayed thus decreasing the overall size and usefulness of the library [1,24]. The advantage of very large libraries is that it is should be possible to isolate antibodies with high affinities, as has been shown by Griffiths et al. [21].

There have been reports using naïve and synthetic libraries for the isolation of scFv or Fab antibody fragments against pure antigens, tumour specific molecules and whole tumour cells. In melanomas, a particularly immunogenic type of tumour, an immunodominant molecule – high molecular weight melanoma associated antigen (HMW-MAA) – has been widely used as a target by a number of groups. Desai et al. [16] reported the isolation of human anti-HMW-MAA scFv fragments by panning a semi-synthetic library (5×10^8 clones) on HMW-MAA purified from Colo 38 melanoma cell lysate by affinity chromatography using murine mAb. scFv 61 immunoprecipitated the > 450 kDa form of condroitin sulphate proteoglycan of HMW-MAA, but not the apparently more immunogenic 250 kDa subunit. Consequently, scFv 61 gave unique information about the structural profile of the HMW-MAA antigen, showing for the first time that the two subunits of HMW-MAA are not associated; these results are in contrast with previous studies using mouse mAb. These finding are supported by a second study, from the same group, when further anti-HMW-MAA scFv fragments were isolated by panning against S5 and SK-MEL-28 melanoma cell lines; a further three antibodies with distinct epitopes were characterised [40]. Comparison of the immunostaining pattern seen on benign and malignant lesions of melanocytic origin with the human scFv antibody fragment 61 and murine mAb showed that the scFv epitope was present in only half of the primary and metastatic biopsies, whereas the mAb recognised over 75%. scFv 61 stained both frozen and formalin-fixed tissue sections, in contrast to the mAb 763.74 that only bound frozen tissue, showed no cross reactivity with guinea pig melanoma and only poor binding to mouse melanoma cells. However, scFv 61 did cross-react with rat neuronal tumour cells, which express NG2, a proteoglycan showing 81% homology in amino acid sequence with HMW-MAA. The novel structural information about HMW-MAA may prove useful in identifying antigenic epitopes for future use as potential immunoadjuvants [16].

Similarly, a synthetic Fab library (Griffith 1, 6.5×10^{10}) was used to isolate human Fab antibody fragments against the c-erbB-2 transmembrane glycoprotein, which is over-expressed in many breast and ovarian tumour cells. The target antigen, together with bound bacteriophage, was isolated using magnetic beads coated with a rat anti-c-erbB-2 specific antibody (ICR55). One of the 9 clones selected and sub-cloned for bacterial expression (B7) was shown to recognise an intracellular epitope both by Western blotting of purified c-erbB-2 and immunofluorescent staining of fixed and permeabilised breast (BT474 and SKBR3) and ovarian (SKOV3) cell lines [44].

Carcinoembryonic antigen (CEA) is the one of the most widely used clinical markers and because of this has been used as a target against which to isolate phage antibodies. Human scFv antibodies against the CEA were isolated from a large human scFv naïve library (2×10^9 clones [41]). One of the antibody fragments

Table 1
Summary of all described clones (groups by tumour type)

Tumour type	Recognised molecule	Phage antibody clone(s)	Selection target	References
Breast	MUC-1	scFv anti-MUC1	MUC-1	Winthrop et al., 1999
Breast	MUC-1	10A, 10B	MUC-1 100mer peptide	Henderikx et al., 1998
Colorectal	(CTAA)28A32-32K	RB15	(CTAA)28A32-32K	Hall et al., 1998
Colorectal	CEA	MFE-23	CEA	Chester et al., 1994
Colorectal	N-terminal of p53		p53	Coomber et al., 1999
Colorectal	CEA	CEA6	CEA	Obsbourn et al., 1996
Colorectal			COLO320, LOVO cell lines	Topping et al., 2000
Colorectal	Ep-CAM	UBS-54	SW180 cell line	Huls et al., 1999
Hodgkin's	CD30	Ki-4	L540 cell line	Klimka et al., 2000
Lung	CD55	LU30	1264, BEAS-2B cell lines	Ridgway et al., 1999
Lung	HuD	GLN495	HuD	Graus et al., 1998
Melanoma	HMW-MAA	K305	Melanoma tissue, melanoma cells	Tordsson et al., 2000
Melanoma		V86	Melanoma cells	Cai et al., 1995;1996
Melanoma	MCSP	E26, G71	A2058 cell line	Cai et al., 1997; Wang et al., 1999
Melanoma	HMW-MAA	scFv 61	HMW-MAA	Desai et al., 1998
Melanoma	HMW-MAA	ScFv#28, #70	S5, SK-MEL-28 cell lines	Nororha et al., 1998
Melanoma	EGF-R	L3 11D, S4 2D	EGF-R	Kettleborough et al., 1994
Melanoma	MAGE-A1	G8	HLA-A1, MAGE-A1	Chames et al., 2000
Melanoma	MAA	LHM2, LHM4	Melanoma cells	Kupsch et al., 1995
Melanoma		A18	A375, DM197 cell lines	Pereira et al., 1997
Ovarian/breast	erbB-2	B7	SKOV3 cell lysate	Sawyer et al., 1997
Ovarian	FBP	C4	OVCAR3 cell line	Figini et al., 1998
Ovarian	mesothelin	SS	mesothelin	Chowdhury et al., 1998
Pancreas	sLe^x, Le^x	S7	sLe^x	Mao et al., 1999
	sLe^x, Le^x	23, 24	Le^x	Dihn et al., 1996
	Fibronectin	CGS-1, CGS-2	ED-B	Carnemolla et al., 1996
	murine VEGF164	V14, V65	Recombinant VEGF164	Vitaliti et al., 2000

that showed a high binding affinity (CEA6) was chosen for the generation of improved affinity variants by mutagenesis and chain shuffling procedures. The derived antibody panel was then incubated with biotinylated CEA in the presence of unbiotinylated CEA for the final wash to enrich for antibodies with a reduced K_{off}. Six antibodies were analysed and shown to have up to 4-fold lower K_{off} rates. Sequencing of the variable genes showed that very few amino acid changes were responsible for differences in the observed dissociation constants for CEA. Such antibodies will allow direct assessment in the correlation between tumour targeting efficiency in relation to binding affinity.

A naïve phage display library containing approximately 6×10^9 different scFv antibodies was used for the isolation of anti-MUC1 specific scFv antibodies [23]. MUC1, a membrane-bound glycoprotein, is over-expressed and under-glycosylated in many human adenocarcinomas of different origins, such as breast, ovary, prostate, and colon. Three different selection approaches were compared for their efficiency of generating anti-MUC1 scFv antibodies: synthetic MUC1-100mer peptides immobilised on immunotubes, biotinylated MUC1 peptide, and a combination of 100mer peptide for the initial 2 rounds and the T47D breast cancer cell line expressing the tumour-associated MUC1 glycoform. The biotinylated MUC1 peptide strategy gave the best results generating a panel of 5 anti-MUC1 scFv antibodies, three of which were subjected to epitope mapping, and shown to recognise different epitopes, that were distinct from the mouse mAb described previously. The two clones which gave the highest absorbance by ELISA, 10A and 10B, were selected for further characterisation by immunohistochemistry and flow cytometry. Both scFv antibodies stained the cytoplasmic and membrane-anchored MUC1 in adenocarcinoma tissues like their murine mAb counterparts, but failed to bind normal tissue expressing MUC1. Flow cytometric studies showed that the antibodies recognised subtly different glycoforms of MUC1, and the conclusion is that clone 10A has potential for use in antibody targeting of breast tumours as it is best able to distinguish between normal and malignant cells. Further studies to assess it's reactivity on a large of panel of breast tissue, normal and malignant, are now required.

The naïve Nissim semi-synthetic scFv phage library has also used for the isolation of antibodies against the ED-B domain in the B-FN splice variant of fibronectin [7]. The B-FN isoform is expressed in the stroma of foetal and neoplastic tissue and in adult and neoplastic blood vessels during angiogenesis, but is

undetectable in mature vessels. The ED-B domain is identical between humans and mice and making it a difficult target to generate antibodies against with hybridoma technology. A range of fibronectin fusion proteins were constructed and were used to select and subsequently characterise the binding domain of the phage antibodies. The isolated antibody fragments, CGS-1 and CGS-2, were compared with the existing mAb BC-1, which specifically recognises the human B-FN isoform at an epitope distinct from the ED-B domain. All three antibodies showed the same immunohistochemical staining pattern on human tissue but the human scFv GCS-2 showed cross-reactivity with other species and supporting the ELISA studies that it recognises an epitope in the ED-B domain, which is highly conserved in different species. This antibody fragment provides a unique tool for the better understanding of the process of angiogenesis during neoplastic development in animal models and is has potential for specifically targeting the neovasculature in humans.

Ridgway et al. [43], isolated human scFv antibody fragments from a naïve library (1.4×10^{10} clones [49]) using live, non-fixed cells from a lung adenocarcinoma cell line (1264) and subtractive panning with a non-tumour epithelial cell line BEAS-2B. The isolated antibody fragments recognise the CD55 antigen, which is overexposed in different tumours and correlates with enhanced resistance to complement-mediated cell lysis *in vitro*. The expression of CD55 antigen in normal lung tissue will most probably exclude the use of the raised scFv antibodies for targeted tumour immunotherapy, however this study highlights two problems with subtractive panning. Firstly, that the removal of all cross-reactive antibodies is very difficult and secondly that cell lines are poor substitutes for fresh cells, i.e. antibodies that react with cell lines of a particular lineage often do not bind to primary cultures or sections of the same tumour type. Both of these problems have been reported also by several other groups [4,5, 46]. A similar study by our group, using a naïve semi-synthetic scFv phage library (5×10^8, Nissim) and a whole-cell panning strategy, selecting on the colorectal cell lines COLO 320 or LOVO and using a two step-subtractive panning, (the opposite colorectal cell line and the breast carcinoma cell line T47D). Some of the isolated scFv antibodies fragments were shown to recognise potentially novel antigens which showed no reactivity against fresh tumours, highlighting the need to use fresh colorectal tumours for the isolation of useful reagents for tumour recognition and possibly targeted immunotherapy [46].

A scFv antibody fragment (UBS-54) was selected from a semi-synthetic phage library (3.6×10^8 clones [15]) by flow cytometry using a colorectal cell line (SW480) as target and the myelomonocytic cell line, U937, as the subtractive panning cell. The isolated scFv antibody fragment was shown to recognise Ep-CAM, a tumour-associated antigen, that has been suggested previously as a suitable target for immunotherapy. scFv UBS-54 was used to construct a human IgG1 mAb, which was shown to mediate efficient killing of tumour cells *in vitro* by both complement- and cell-dependent mechanisms. The use of fully human mAb have some advantages over scFv or Fab: increased serum half-life, bivalency and therefore increased avidity, and effective recruitment of leucocyte effectors [28].

Furthermore, phage display technology has been used successfully for the production of bispecific antibodies by the fusing of two human scFv fragments. An scFv fragment (NM3E2) that binds specifically to CD16 was fused with an anti-HER2/neu scFv (C6.5) creating a bispecific scFv antibody. Both antibodies were derived from naïve scFv libraries using recombinant material and possess dissociation constants $> 10^8$ M^{-1} ([37,45] respectively). The bispecific antibody was effective in mediating redirected cellular cytotoxicity of SK-OV-3 ovarian tumour cells by PBL, and biodistribution studies in SCID mice bearing SK-OV-3 gave favourable results, i.e. high tumour: organ ratio. These results suggest that bispecific scFv fragments generated from naïve libraries have potential as selective targeting agents, similarly there would be no reason why antibody fragments generated from immunised libraries also could not be used.

Recently, Chames et al. [8] have generated phage antibodies that specifically recognise an antigenic peptide associated with an MHC class I molecule. HLA-A1-MAGE-A1 is a well-characterised T cell epitope found in many tumours. One of the isolated antibodies (G8) specifically recognised the HLA-A1-MAGE A1 but not the HLA-A1-MAGE A3 complex. The antibodies are reactive with a tumour T cell epitope, in association with HLA-1, and will be extremely useful in dissecting the cytotoxic T lymphocyte (CTL) repertoire and possibly as targeting reagents for combined antibody and CTL immunotherapy. This study used biotinylated, recombinant HLA-1-peptide as the target, which after incubation with the phage library, were isolated with streptavidin-coated magnetic beads. The fact that these antibodies were isolated from a large, naïve Fab phage antibody library (3.7×10^{10} clones) demonstrates the

diverse specificities contained within such libraries. It remains to be established whether antibodies for any HLA-peptide can be isolated from all naïve libraries.

Recently scFv antibodies have been described that block tumour angiogenesis in a mouse model by binding vascular endothelial growth factor [50]. Antibodies were isolated from the naïve, Nissim scFv library by selection against immobilised recombinant mouse $VEGF_{164}$. Two antibodies, V14 and V65, reproducibly caused inhibition of angiogenesis in a standard chorioallantoic membrane assay. Furthermore, daily administration of clone V65 significantly inhibited the growth of subcutaneously injected FE-8 tumour cells in a nude mouse model. Epitope mapping of the clones was not performed but the encouraging *in vivo* results mean that it is proposed to generate higher affinity reagents for further studies.

3. Phage antibodies from immunised libraries

Immunised phage display libraries are most often generated from V-genes derived from IgG mRNA of B cells of an immunised animal or human [11]. Consequently, these libraries are enriched with genes encoding variable domains of antibodies specific for the desired antigen target [1,53]. A number of methodological papers have been published, those by Mutuberria et al. [38] and Hoogenboom et al. [25] provide an excellent and up to date overview.

A number of studies have used immunised libraries against colorectal cancer. MFE-23 is one of the best characterised scFv antibodies that was isolated from a phage library (1×10^7 clones) generated from mice immunised with human CEA [9]. This clone showed the same staining pattern as existing anti-CEA mAb being positive on human colorectal adenocarcinomas with little cross-reactivity to various normal tissues. A full discussion of this antibody and its use *in vivo* is given by Chester and co-workers elsewhere in these conference proceedings. A second antigen that is thought of as a potential target for detection and treatment of various solid tumours, including colorectal, is Ep-CAM, also known as EGP-2, KSA and 17-1A antigen. Phage display technology was used to generate new antibodies with enhanced binding characteristics for use *in vivo*, based on existing murine mAb sequences. The kinetics of four well-characterised anti-Ep-CAM mAb (17-1A, 323/A3, MOC-31, and MOC-161) were studied and it was shown that MOC-31 and MOC-161 possess the lowest apparent off-rates. The V genes from these two antibodies were cloned in a scFv format in a phage display vector (pCANTAB6) and antigen binding phage were isolated by panning on immobilised, baculovirus-expressed, recombinant Ep-CAM. A relatively small library (10^6 clones) from MOC-31 gave 15 positives from 90 clones analysed by ELISA after two rounds of selection. Two clones selected for further analysis, on the basis of different DNA sequences, retained the same immunohistochemical staining pattern with malignant and normal tissue as their mAb ancestors but they possessed significantly lower off-rates making them better tools for tumour-targeting. Further work by this group have used the technique of "guided selection" to produce a fully human antibody possessing the specificity and high-affinity of MOC-31. To do this variable genes from MOC-31 were cloned into the phagemid vector pCES1 which produces a Fab library. After two rounds of chain shuffling, introducing first human V_L and then V_H sequences isolated from spleen cells, Fab antibodies were selected on soluble, biotinylated EP-CAM under stringent conditions [3]. The best clone, 9E, behaves just like MOC-31 in ELISA, flow cytometry and immunohistochemistry although the chemistry of the human antibody-antigen interaction may be different. The subtle differences in the chemistry require that antibodies generated in this way must be screened for such functions as antibody-induced signal transduction.

The "guided selection" technique described above was also used to generate human Fab antibodies against the α isoform of folate-binding protein (FBP), a cell surface antigen which is over-expressed on approximately 90% of ovarian carcinomas and is associated with disease expression. In this case the chain-shuffling order was reversed; human V_H and then V_L fragments were introduced, with selection being performed on glutaraldehyde-fixed ovarian carcinoma cells (OVCAR3). The mouse mAb, Mov 19, was used as the initial template. Although the best clone, C4, was highly specific for FBP, as shown by immunoprecipitation and ELISA, it had a fivefold lower affinity for OVCAR3 cells than the mAb Mov 19. One interesting facet of this Fab fragment is that it was also shown to bind to a cryostat section from a FBP-over-expressing ovarian carcinoma, even though it was selected against fixed target cells. As this results were obtained using a single tumour, further work is required to establish whether the cross-reactivity between fixed and frozen targets is due to the FBP molecule or a property of the guided selection technique [18].

A second molecule that has been identified on a number of tumours, including ovarian carcinoma, is the

40 kDa glycosylphosphatidylinositol-linked glycoprotein, mesothelin [10]. A scFv phage display library (9×10^5 clones) was made from splenic mRNA from mice immunised with Swiss NIH3T3 cells transfected with the full-length mesothelin gene. scFv antibody fragments were isolated against immobilised recombinant mesothelin with three rounds of selection. A single scFv clone, SS, that was shown to be specific for mesothelin was used to construct an immunotoxin by fusion with a truncated mutant of Pseudomonas exotoxin A. The immunotoxin was shown to be highly effective and specific in nude mouse and *in vitro* assays in lysing mesothelin positive cells. This antibody and related fusion products are excellent candidates for use *in vivo*, particularly after some form of humanisation.

Not surprisingly most of the antibodies isolated to date react against cell surface expressed molecules, but phage display libraries have also been used to generate tools against intracellular molecules. Recently, individual Fab phage display libraries were constructed using lymph node tissue from six patients with colorectal cancer, all of whom had a demonstrable serum response against p53. The libraries varied in size from $1.3 \times 10^6 - 4.5 \times 10^7$ clones. After panning against recombinant p53 coated on plastic 14 anti-p53 specific antibodies were isolated. Four of these showed high affinity ($> 10^{-8}$ M) to recombinant wild-type p53 and a mutant form immunoprecipitated from colorectal cells. The antibodies bound to the amino-terminal region of p53, between residues 27 and 44, a region to which few mAb have been described previously [13]. These reagents will aid our understanding of this facet of the immune response.

Similarly, a Fab phage library (2×10^7 clones) constructed using variable region genes extracted from a tumour-associated lymph node of a colorectal patient. It was panned for reactivity with the extracellular domain of HEK-293 expressed c-erbB-2. Sixteen independent antibodies, representing 4 different V_H families, specific for c-erbB-2 were successfully isolated. The V gene usage of the isolated anti-c-erbB-2 Fab fragments suggested that they were probably the result of the patient's humoral response, and indicated that *in vivo* antibody response to tumour-associated antigens could lead to the *in vitro* production of tumour specific recombinant antibodies [12].

Rather than using lymph node cells as the source of antibody genes, Hall and colleagues have used peripheral blood lymphocytes from patients undergoing active specific immunotherapy (ASI) with autologous carcinomas (22 colon, 2 kidney, 1 ovary, 1 lung and 1 squamous, together with an adjuvant); two scFv phage display libraries were created (3.1×10^7 and 2×10^8 clones). Both libraries were panned against the colon tumour associated antigen CTAA 28A32-32K which is related to the annexin family of proteins and has been shown previously to elicit potent T cell mediated responses. In a similar study to that of Henderikx et al. [23] different selection methods were used and once again the use of biotinylated target appeared to be an important component of a successful panning strategy. A single scFv, RB15, was isolated that reacted specifically with CTAA 28A32-32K and was shown to have similar binding specificity to the murine hybridoma-derived 5-11A scFv, recognising an epitope at the amino terminus. Conversion of the RB15 into a IgG1 molecule gave a human antibody with identical binding properties [22]. It is likely that these ASI libraries will allow the identification of other tumour-specific scFv, against a wide variety of tumours, and perhaps also the isolation of novel tumour associated-antigens.

Another example of phage library technology helping the understanding of immune response is in the autoimmune aetiology of paraneoplastic encephalomyelitis and sensory neuropathy (PEM/SN) that is associated with small-cell lung cancer (SCLC). SCLC express the HuD antigen, a member of a conserved family of neuronal RNA binding proteins, and patients with high anti-Hu titres develop PEM/SN. A Fab phage display library (3×10^6 clones) was constructed from mRNA extracted from metastatic lymph node from patients with SCLC. Immobilised, purified recombinant HuD protein was used for antibody selection with increasing wash stringency. One of the antibodies, Fab GLN495, reacted in immunohistochemistry and Western blot with SCLC and neurones. The antibody inhibited up to 75% of the anti-Hu antibodies of the patient from which it was derived and competed with most anti-Hu sera from patients with PEM/SN suggesting that it recognises an immunodominant epitope. Such antibodies could also be useful in the diagnosis of HuD expressing tumours [20].

Growth factor receptors comprise a large family of molecules some of which also show enhanced expression on a variety of malignant tissues; probably the best-studied is the epidermal growth factor receptor (EGFR). Kettleborough and colleagues have undertaken a comparative study of mouse anti-EGFR antibodies isolated from various libraries with a hybridoma produced mAb, 425. Three different scFv libraries were constructed using different sources of mRNA: the spleen of a mouse immunised with A431 cells (8.8×10^5 clones);

the popliteal lymph node of a mouse immunised in the footpad with purified, recombinant human EGFR (6.5×10^6 clones); and from mouse cells immunised *in vitro* with A431 vesicles (1.1×10^5 clones). All the libraries were relatively small in size compared with the naïve semi- and completely synthetic versions described above but it is clear that novel, useful reagents can be isolated from these "biased" repertoires. The best library in terms of fewest selection steps, greatest diversity and largest number of high affinity antibodies isolated was the one generated using the draining lymph node [29]. Four scFv antibody fragments were identified that bound both purified EGFR and EGFR-bearing tumour cells by ELISA, but only two (L3 11D, S4 2D) bound to A431 cells using flow cytometry and these gave similar profiles to the hybridoma-derived scFv 425. Furthermore, the two purified scFv molecules were converted into whole antibodies by joining the mouse variable regions to human constant regions to create partially humanised chimeric IgG antibodies and these retained similar binding profiles and affinities to the mouse 425 mAb.

Aberrant glycosylation of surface proteins is a common feature in malignancy and targeting of such molecules has been the subject of much interest over many years for detection, staging and immunotherapy. One strong candidate is the MUC-1 mucin, a molecule abundantly expressed on breast cancer as well as other cancers of epithelial origin (prostate, colon, lung, breast, and ovary), and although present in normal epithelial cells modifications in its glycosylation by cancer cells produce atypical sugar structures. A panel of anti-MUC-1 scFv, recognising a wide range of epitopes, was isolated from a scFv phage display library constructed from V-genes of mice immunised with MUC-1 antigen isolated from MCF-7 and HBT 3477 human breast carcinoma cell lines [54]. Phage were selected using decreasing concentrations of biotinylated MUC-1 and a library containing greater than 5×10^5 clones was generated. A number of clones were shown to bind well to MCF-7 membranes by ELISA, but no data as to which epitopes were recognised or if such antibodies would react with fresh tumour tissue. As discussed above, cell lines appear to make poor substitutes for fresh tissue, therefore it will be interesting to see if this is true for studies using an immunisation strategy against purified molecules.

It is well known that generating high affinity anti-carbohydrate antibodies using the hybridoma method is difficult as the nature of the antigen drives a response that produces IgM antibodies. It is becoming increasingly clear that carbohydrate-lectin mediated adhesion is an important event with molecules such as sialyl Le^x being involved in tumour cell-endothelial cell interactions and in extravasation during metastasis [35]. Dinh and colleagues [17] have reported the isolation of anti-Le^x and anti-sialyl-Le^x reactive antibodies using a Fab phage library (approximately 3×10^6 clones) constructed from mRNA of mice immunised with GM1 ganglioside. Antibodies were selected against immobilised Le^x-BSA with four rounds of panning. The two selected antibodies (clone 23 and 24) not only had better affinity for the Le^x and sialyl-Le^x antigens in comparison with the hybridoma mAb, but they also recognise distinct, possibly non-overlapping epitopes [17]. More recently, Mao et al. [33] isolated anti-Le^x and anti-sialyl-Le^x antibodies from a human scFv phage library (approximately 10^8 clones), constructed using PBL from twenty different cancer patients. Eleven different tumours including both leukaemias and carcinomas were represented. Selection was performed using decreasing concentrations of immobilised sialyl- Le^x with four rounds of panning. Four scFv antibody fragments (S6, S7, S8, and S10) were isolated and shown to have similar affinities which varied between 1.1 and 6.2×10^{-7} M for Le^x and sialyl-Le^x. Detailed analysis of the antibodies' cross-reactivity with other glycolipids or synthetic analogues was not performed, but BIAcore binding assays and flow cytometry experiments using the pancreatic adenocarcinoma cell line SW1990 suggest that the clones bind distinct epitopes. In summary, there is still no evidence that any phage derived anti-carbohydrate antibody reacts with fresh tumour tissue; a question that needs to be addressed urgently.

As mentioned above a considerable amount of work has focussed on melanoma. The isolation of human anti-MAA (melanoma associated antigen) scFv and Fab fragments has been reported by panning on cultured melanoma cells. Both libraries were constructed from peripheral B lymphocytes taken from patients with malignant melanoma who were immunised with either a vaccinia virus melanoma oncolysate (10^8 Fab-expressing clones; [42]) or interferon γ transfected melanoma cells ($4 \times 10^7 - 5 \times 10^8$ scFv; [6]). A similar whole cell panning strategy was adopted by both groups incorporating a negative selection step with normal melanocytes in an attempt to remove antibodies that recognise epitopes on normal tissue. From the panels of antibodies that were isolated each group focussed more detailed study of their best clone. One novel aspect of the work by Pereira et al. [42] is that the antibody was selected without amplification, i.e.

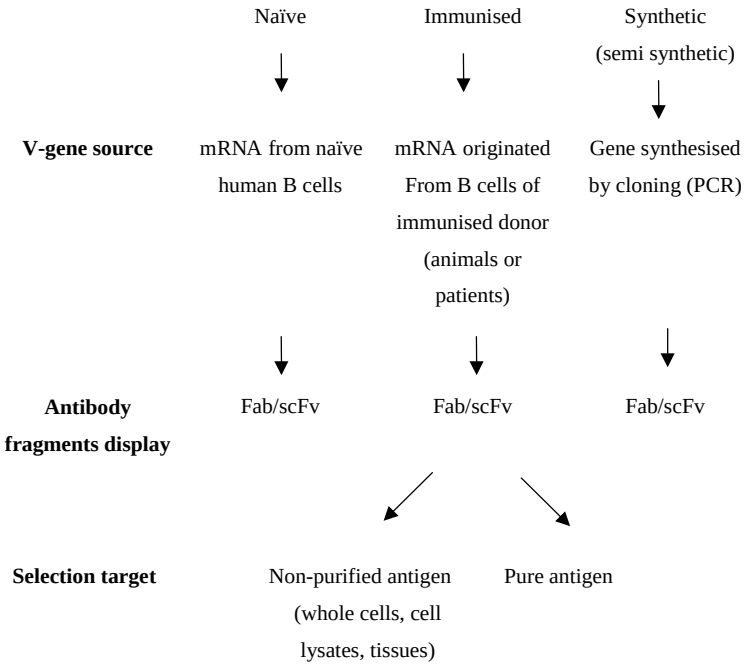

Fig. 1. Overview of the different phage antibody display libraries.

the adsorption and positive selection were immediately followed by isolation of individual clones. This means that there is no selection of phage with a growth advantage but also means the beneficial aspects of point mutations and resulting increase in affinity with progressive rounds of selection do not occur. One Fab antibody, A18, was shown to bind strongly to normal nevus and metastatic melanoma cells, with weak reactivity to a number of other tissues. Obviously this antibody would have little use for targeting *in vivo*, but again demonstrates that self-reactive antibodies can be generated using phage antibody display libraries [42]. Cai and Garen reported a scFv antibody fragment (V86) that showed reactivity by cell ELISA and immunohistochemistry to melanoma biopsy and metastatic lesions with no staining of normal epithelial or other carcinoma tissues [6]. This clone was examined further and found to be truncated, with most of the V_L domain had been lost during construction. It was also subsequently shown that addition of a V_L partner to the V86 heavy chain reduced or eliminated the binding capacity of V86 to melanoma cells [5]. A phage library was prepared expressing single V_H domains by Cai and Garen and it was shown that a number of different V_H fragments could be isolated from such a library, proving that the V86 clone was not an artefact [4]. The importance of the V_H domain was demonstrated previously by the studies of Ward et al. [52] and therefore single libraries of single V_H domains may also be useful tools for isolating tumour-specific reagents, applications to other tumours has not yet been done. Two of the isolated scFv, (G71 and E26), that recognised a 250 kDa melanoma-associated chondroitin sulphate proteoglycan, were fused with the immunoglobulin Fc domain to construct an immunoconjugate that effectively mediated NK and complement lysis of melanoma cells in vitro [51]. The *in vivo* applicability of such reagents remains to be demonstrated, but these studies clearly show the progression from interesting scFv to possible therapeutic reagent.

Kupsch and colleagues generated an scFv library (9.3×10^7 clones) using peripheral blood lymphocytes from 10 human donors that showed high autoantibody titre against two melanoma cell lines. Two antibodies, B3 and B4, were isolated by a standard whole cell selection strategy using a melanoma cell line and peripheral blood lymphocytes as a negative selection step. Flow cytometry, ELISA and immunohistochemistry were used to show that the antibody did not bind to normal tissue but recognised epitopes that were present on other tumours in addition to melanoma [31]. These two antibodies showed considerably less non-specific binding than a previously reported murine mAb LHM2, which reacted with high molecular weight (HMW) proteoglycan, that had also been expressed as a phage-expressed scFv [32]. The molecules to which B3 and

B4 bind are not known but because of differences in immunohistochemical and flow cytometric staining patterns to LHM2, they are thought not to bind HMW proteoglycan. The high degree of tumour-specificity gives these reagents many potential clinical applications.

Tordsson and colleagues have isolated an scFv antibody fragment specific for the immunodominant HMW-MAA, using a phage antibody library derived from a human melanoma-immunised cynomolgus monkey. Lymph node cells were used as the source of mRNA and a scFv library containing 3×10^7 clones was generated. Selection was performed either on acetone-fixed tissue sections (methodology described in detail in [48]) or whole tumour cells [47]. The primate K305 scFv fragment had an affinity of 1.6×10^{-9} M for HMW-MAA by Scatchard analysis and showed restricted cross-reactivity with normal smooth-muscle cells. The K305 was then expressed as a Fab fragment and fused to a mutant form of the staphylococcal enterotoxin A superantigen. Redirection of T cell cytotoxicity to melanoma cells *in vitro* as well as T-cell dependent growth inhibition of established human melanoma tumours in a SCID mouse model were demonstrated, using the K305-SEA fusion protein [47]. As primate antibodies have a high degree of homology with human species the problems of a neutralising response should be diminished. The fusion with SEA offers a way of boosting the T cell immune response in a tumour-targeted manner; clinical trials with such reagents are awaited.

The CD30 antigen is expressed on Hodgkin-Reed Sternberg cells, characteristic of primary Hodgkin's lymphoma, in addition to a number of other tumour cells. Recently, Klimka et al. [30] have taken the V genes from the murine anti-CD30 mAb Ki-4 and have generated an scFv library. After selection on the CD30-positive cell line L540, a scFv was isolated that bound to an epitope that overlapped with that recognised by Ki-4. A recombinant fusion-protein with Pseudomonas exotoxin was prepared and shown *in vitro* to inhibit protein synthesis at an IC50 of 43 pM. More recently this group have used guided selection on the mAb Ki-4 followed by selection on L540 cells to produce a fully human scFv, as described above by Beiboer et al. [3]. This scFv antibody had almost identical properties to the parental mAb and murine scFv, in particular it could inhibit shedding of the extracellular domain of CD30, an important point if CD30 is being used as a target molecule. The only major difference observed was a drop in apparent affinity from 5×10^{-9} to 3×10^{-8} M for Ki-4 Fab and human Ki-4 scFv, respectively. Even so, the affinity for this antibody is in the nanomolar range and as it possesses all the qualities of the parental mAb it is a very promising candidate for immunotherapy, either alone or as an immunotoxin.

4. Conclusion

As we shown above a large number of novel antibodies have been generated using phage antibody display technology. A number of issues remain to be addressed including the relationship between the form of the selecting target and final reactivity and hence usefulness of the isolated clones. A simple rule is to select against targets in the same form as one wishes to use the specific clones. With the exception of MFE-23 few antibody fragments have been used widely *in vivo*, and it is this step that must be now taken for a variety of different products: antibody fragments alone, bispecifics, toxin-, radio-isotope- or superantigen-fusion proteins, etc. The application of phage antibodies in cancer alone is vast and the coming five years are likely to see the arrival of some clinically useful tools.

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