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Isolation of human anti-c-*erb*B-2 Fabs from a lymph node-derived phage display library

M. A. CLARK*, N. J. HAWKINS‡, A. PAPAIOANNOU*†, R. J. FIDDES†§ & R. L. WARD*†
Department of Medical Oncology, St Vincent's Hospital and †CRC for Biopharmaceutical Research, Darlinghurst, ‡School of Pathology, University of NSW, Sydney, and §Garvan Institute of Medical Research, Darlinghurst, Australia

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SUMMARY

An immunoglobulin phage display library constructed from a tumour-associated pericolic lymph node was panned against the extracellular domain of the oncoprotein c-*erb*B-2. Sixteen independent clones were confirmed as positive binders based on ELISA analysis of soluble Fabs. Nucleotide sequencing demonstrated that the V_H region of 12 clones belonged to four different V gene families, and the clones demonstrated varying degrees of somatic mutation compared with germ-line sequences. Fab fragments were examined for cross-reactivity by ELISA and shown to be negative against a panel of irrelevant self and non-self antigens, including bovine serum albumin (BSA), mouse immunoglobulin, tetanus toxoid, heregulin-PE40-FLAG and insulin. Reactivity of Fabs *in vitro* was verified by immunocytochemistry, which showed binding to the c-*erb*B-2 over-expressing breast cancer cell line SKBR3 but not to the low-expressing cell line MDA-MB-231. We conclude that a single lymph node library of moderate diversity (2×10^7 κ light chain and γ heavy chain clones), when derived from an individual whose colorectal tumour over-expressed c-*erb*B-2, can be successfully panned to isolate a number of unique Fabs specific for this antigen. The nature of the anti-c-*erb*B-2 Fabs recovered from this library suggests that they may have resulted from a humoral immune response in the individual, and that *in vivo* antibody responses to tumour-associated antigens may be exploited *in vitro* for the production of tumour-specific recombinant antibodies.

Keywords c-*erb*B-2 phage display Fabs human anti-tumour antibodies

INTRODUCTION

Combinatorial antibody libraries, in which paired light and heavy chain variable regions are displayed on the surface of filamentous bacteriophage, are widely used for the isolation of human antibodies with specificity for haptens, foreign antigens and self antigens. The theoretical advantages of recombinant human antibodies over mouse monoclonals as therapeutic agents are well recognized [1–5]. However, the optimal source of immunoglobulin genes for the construction of these libraries remains uncertain.

Phage display libraries have been constructed from immunoglobulin genes derived from a wide variety of tissues, including peripheral blood, bone marrow and lymph nodes [2,6,7]. These tissues are frequently obtained from immunologically naive, healthy donors [5,6]. The resultant non-immune libraries have proved useful sources of functional antibodies to a variety of antigens. Moreover, it has recently been demonstrated that the retrieval of specific antibodies can be enhanced by increasing the library size and by using synthetic immunoglobulin genes [3,4,8]. The diversity and size of synthetic libraries, and thus the affinity of particular antibodies, can be further increased by combinatorial infection and *in vitro* recombination strategies [3,9,10]. However, this artificial construction of large non-immune libraries is technically challenging and introduces a number of potential problems. For example, since the antibody sequences have not been selected *in vivo*, a large percentage are likely to be non-functional. More importantly, selected antibodies may prove strongly immunogenic or demonstrate critical cross-reactivity *in vivo*.

An alternative and valuable source of mRNA for the construction of phage display antibody libraries is lymphoid tissue from individuals who, because of disease, have mounted an immune response to disease-related antigens [2,11]. A humoral immune response to altered or over-expressed tumour-related antigens such as p53 [12,13] and c-*erb*B-2 [14,15] has been demonstrated in some patients with cancer. These individuals may provide an enriched source of disease-related immunoglobulin genes, and therefore libraries derived from them may contain a larger pool of relevant antibodies which can be recovered by panning against

Correspondence: Dr Robyn Ward, Department of Medical Oncology, St Vincent's Hospital, Darlinghurst, NSW 2010, Australia.

specific tumour antigens. Moreover, the examination of Fabs isolated from such libraries may provide insights into the nature of the immune response to cancer.

This study describes the isolation of functional Fabs to the surface glycoprotein c-*erb*B-2 from one such 'immune' library after panning. Several Fabs demonstrated *in vitro* binding to the breast cancer cell line SKBR3 which over-expresses the c-*erb*B-2 antigen. These results support the use of naturally occurring immunoglobulin genes, selected by the donor's immune system, in the construction of phage display libraries.

MATERIALS AND METHODS

*Production of the extracellular domain of c-*erb*B-2*

The cDNA of the extracellular domain (ECD) of c-*erb*B-2 (aa 1–650) was amplified by polymerase chain reaction (PCR) from the plasmid pSVc-*erb*B-2 which contains the full-length cDNA of human c-*erb*B-2 (gift of M. Hibbs, Ludwig Institute for Cancer Research, Melbourne, Australia). The forward primer (nt 170–186) of the c-*erb*B-2 open reading frame contained an *Xba* I site, while the reverse primer introduced a stop codon and a *Bsp* EI site after aa 650. The cDNA coding for the ECD was cloned into pRc/CMV (Invitrogen, San Diego, CA) which had been modified to encode the FLAG epitope DYKDDDDK (pRc/CMV_{FLAG}). Constructs were confirmed by DNA sequencing.

Human embryonic kidney cells HEK-293 were transfected with c-*erb*B-2-pRc/CMV_{FLAG} by calcium phosphate co-precipitation. Transfected cells were incubated in selection medium containing G418 (500 μg/ml active geneticin; GIBCO, Grand Island, NY), stable transfectants were cloned and c-*erb*B-2 expression in the medium was monitored by immunoblotting using antibodies specific for either ECD-c-*erb*B-2 (Santa Cruz Biotechnology Inc.) or the FLAG epitope (M2 anti-FLAG antibody; IBI, New Haven, CT). Pure preparations of c-*erb*B-2 were obtained by affinity chromatography using M2, their purity was assessed by SDS–PAGE and their immunoreactivity determined by immunoblotting.

Construction and panning of the phage library

An IgG1 κ library (LNM) containing 2×10^7 κ light chain and γ heavy chain clones was constructed in vector MCO1 [7]. Total RNA was isolated from a pericolic lymph node of a woman with colorectal cancer. Preparation of phage and panning were performed as previously described [7]. Wells of a microtitre plate (Nunc, Immunosorp, Roskilde, Denmark) were coated with c-*erb*B-2 (10 μg/ml) overnight at 4°C in carbonate buffer pH 9·6 and blocked with 200 μl 2% skim milk powder in PBS (2% MPBS) for 2 h at 37°C. A 100-μl aliquot of the library phage ($1–2\times10^{12}$ colony-forming units (CFU)) was added to each well with 100 μl 2% MPBS and the plate was incubated for 2 h at room temperature. Each well was washed 20 times, first with 200 μl PBS/0·1% Tween 20 and then with PBS. Bound phage were eluted with 100 μl 0·1 M glycine–HCl pH 3·0 and used to infect logarithmic phase ($A_{600}\approx0{\cdot}8$) *Escherichia coli* XL1-Blue. Panning was continued for three rounds as previously described.

Analysis of phage binding by ELISA

Phage clones from output round 3 of panning were grown overnight in 200 μl 2×YT/1% glucose/50 μg/ml carbenicillin (2YT/glu/carb) in wells of a microtitre plate. A small inoculum (5 μl) from each well was transferred to a new plate containing 200 μl 2YT/glu/carb and grown with shaking for 1 h at 37°C. Helper phage (10^9 plaque-forming units (PFU) VCS-M13; Stratagene, La Jolla, CA) in 25 μl broth were added to each well, left standing for 30 min at 37°C, then incubated for an additional 1 h at 37°C. Cells were pelleted (1700 ***g***, 10 min) then resuspended in 200 μl 2YT/carb containing 70 μg/ml kanamycin and grown with shaking overnight at 30°C. Cells were pelleted again and the supernatant was transferred to a microtitre plate which had been coated with c-*erb*B-2 and blocked as described above. After washing extensively, primary antibody (100 μl biotinylated sheep anti-M13, 1:1000 in 2% MPBS; 5′-3′) was added and incubated for 1 h at room temperature. Streptavidin-alkaline phosphatase (SA-AP, 100 μl, 1:1000 in 2% MPBS; Jackson, West Grove, PA) was added to the wells and incubated for 20 min at room temperature. The substrate *p*-nitrophenyl phosphate was added and absorbance at 410 nm (A_{410}) determined. Clones were considered positive for c-*erb*B-2 if they gave a signal at least four-fold higher than either irrelevant control antigen (bovine serum albumin (BSA) at 10 μg/ml) or no antigen. Monoclonal anti-c-*erb*B-2 (1:1000 in 2% MPBS; Santa Cruz Biotechnology Inc.) was included in ELISA analyses as a positive control.

Preparation and screening of soluble Fabs

Log phase *E. coli* HB2151 (1 ml) were infected with phage ($\approx 1–5\times10^{11}$ CFU) by incubation for 30 min at 37°C without shaking. An aliquot was then removed, serially diluted in 2YT, plated onto LB agar/carb containing 1% glucose and incubated overnight at 37°C. Single colonies were inoculated into 100 μl of 2YT/glu/carb in wells of a microtitre plate and grown with shaking for 16 h at 37°C. A 10-μl aliquot of each culture was then added to 200 μl of 2YT/carb containing 0·1% glucose and grown with gentle shaking at 37°C for a further 3 h or until $A_{600}\approx0{\cdot}8$. Isopropylthiogalactoside was added to a final concentration of 1 mM and cultures were grown with shaking at 30°C for 16–18 h or 5 h if the periplasmic fraction was to be prepared [16]. Cells from overnight cultures were pelleted and 70 μl of supernatant were transferred to an ELISA plate which had been coated with c-*erb*B-2 and blocked as described.

Wells were washed, then incubated with 100 μl sheep anti-human κ-chain antibody (1:100 in 2% MPBS; The Binding Site, Birmingham, UK) for detection of the light chain, or anti-*myc* 9E10 MoAb (1 μg/ml in 2% MPBS [17]) for detection of the heavy chain for 1 h at room temperature. After washing again, 100 μl donkey anti-sheep horseradish peroxidase (HRP) conjugate (1:500 in 2% MPBS; Silenus, Hawthorn, Australia) or goat anti-mouse (Fc-specific)-AP (1:1000 in 2% MPBS; Jackson) were added for a further 1 h at room temperature for detection of light and heavy chains, respectively. Following washing five times with PBS, the appropriate chromogenic substrate was added and the absorbance was determined. Fabs were also tested for binding to tetanus toxoid (TT; CSL, Melbourne, Australia), BSA (Sigma, St Louis, MO), insulin (Novo Nordisk, Sydney, Australia), mouse immunoglobulin (Becton Dickinson, Mountain View, CA) and a recombinant FLAG-tagged protein (heregulin-PE40-FLAG [18]). All antigens were coated at 10 μg/ml overnight at 4°C in carbonate buffer pH 9·6.

DNA fingerprinting and sequencing of clones

Plasmid preparations were subjected to *Bst* NI digestion [19] to identify unique clones. Selected clones were sequenced on at least two occasions with Taq dideoxy chain terminators and run on a

373 A Automated Sequencing System (Applied Biosystems, East Kew, Australia) using a pelB primer (5′-gcc tac ggc agc cgc tgg-3′) for the heavy chain variable region. Sequences were analysed by reference to the Kabat Database [20] and V BASE Sequence Directory (Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, UK).

SDS–PAGE and immunoblotting

Culture supernatants or periplasmic fractions of Fab fragments (20–50 μl) were subjected to 10% SDS–PAGE, then either electroblotted onto nitrocellulose membrane (0·45 μm; Nitrobind, MSI, Westboro, MA) overnight at 4°C or stained with coomassie blue R-250. Membranes were blocked for 1 h in 2% MPBS at room temperature and κ light chains were detected by chemiluminescence (ECL) using primary and secondary antibodies as described for assay of soluble Fabs. ECL substrate was used according to the manufacturer's directions (DuPont-NEN, Boston, MA). Gamma heavy chains were detected as described for soluble Fabs.

Immunohistochemistry

For determination of c-*erb*B-2 expression on the primary tumour, 4-μm paraffin sections were rehydrated, microwaved in 0·01 M citrate buffer pH 6·0 for 3 min and then blocked with normal goat serum (1:5 in Tris-buffered saline (TBS)) for 20 min. Sections were then incubated for 1 h at 37°C with rabbit anti-c-*erb*B-2 antibody (1:100 in TBS/2% BSA; Dako, Glostrup, Denmark), washed, and bound antibody detected by incubation for 30 min with a biotinylated goat anti-rabbit antibody (1:300 in TBS/2% BSA; Vector Labs, Burlingame, CA) followed by streptavidin–HRP (1:500 in TBS/2% BSA; Vector Labs). Colour was developed with diamine benzidine tetra hydrochloride (0·03% in 0·003% H_2O_2) and sections were lightly counterstained with haematoxylin before mounting.

For immunocytochemistry, human breast adenocarcinoma cell lines either over-expressing c-*erb*B-2 (SKBR3; ATCC no. HTB-30) or expressing very low amounts of c-*erb*B-2 (MDA-MB-231; ATCC no. HTB-26) were used. Cells were smeared on silane-coated slides and fixed in cold acetone for 10 min. Before use, slides were incubated with 3% H_2O_2 for 5 min, then blocked with normal rabbit serum (1:5 in TBS; Vector Labs) for 20 min at room temperature. Fabs (1:3 in TBS/2% BSA) were added for 1 h at 37°C, the slides were washed in TBS and bound Fabs were detected by incubation for 1 h with 9E10 antibody (1:100 in TBS/2% BSA). Biotinylated rabbit anti-mouse (1:300 in TBS/2% BSA; Dako) was incubated on slides for 1 h followed by streptavidin–HRP (1:100 in TBS/2% BSA; Dako) for a further 1 h at room temperature. Colour was developed as described above.

RESULTS

After three rounds of panning, 24/96 clones were found to be reproducibly reactive with c-*erb*B-2 by phage ELISA ($A_{410} > 0·8$). Following *Bst* NI fingerprinting and analysis of soluble Fabs by ELISA, 10/24 clones from output 3 (3A2, 3B3, 3E2, 3E3, 3E9, 3E10, 3E12, 3F2, 3H7, 3H11) and 6/96 clones from input 4 (4A2, 4C4, 4C5, 4D2, 4D4, 4F7) showed a unique restriction pattern and reproducible reactivity against c-*erb*B-2. These clones showed no cross-reactivity against TT, BSA, insulin or heregulin-PE40-FLAG as assessed by Fab ELISA (Fig. 1).

The deduced amino acid sequences of 12/16 clones are shown in Table 1. Comparison with the Kabat database and the V BASE Sequence Directory indicated that three different V_H gene families were used, with 7/12 being V_H4, 4/12 being V_H3, and 1/12 being V_H1 (Table 2). Most V_H genes had several differences from the nearest germ-line V gene, suggesting that they were from substantially mutated B cells. The degree of somatic mutation was highly variable, with the CDR1 and CDR2 regions of one clone (3A2) being close to germ-line, whilst another clone (3F2) from the same germ-line family showed considerable somatic mutation. In the V_H4 family, two clones (3A2 and 3F2) were related to the DP-65 germ-line gene, whilst another two clones (4A2 and 4C5) had closest homology to the M95144 germ-line gene.

Although these Fabs were expressed at levels readily detected by dot blot (data not shown), their expression was variable, in that component light and heavy chains could only be detected in ≈50%

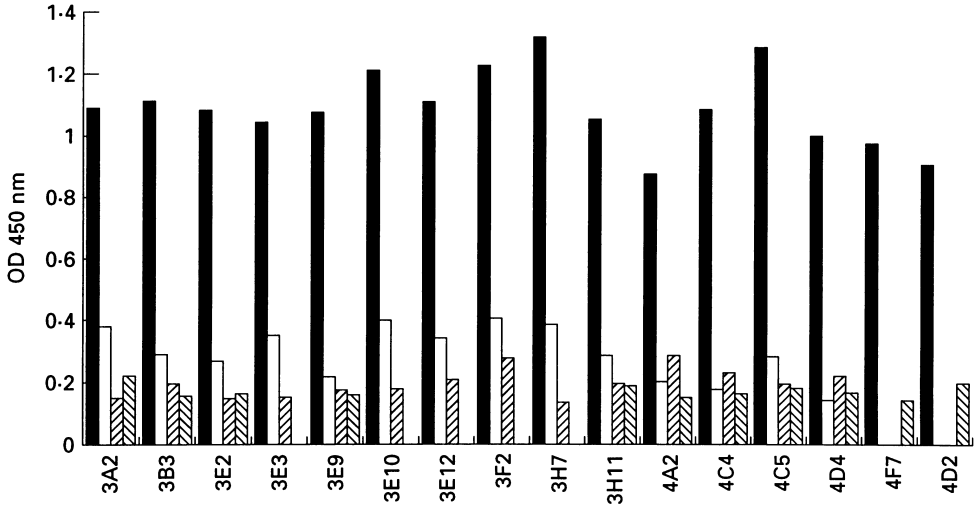

Fig. 1. Specificity of soluble anti-c-*erb*B-2 Fabs. Binding was determined by ELISA to a range of antigens. Results for clones 3A2, 3B3, 3E2, 3E9, 3H11 and 4C5 against c-*erb*B-2 and tetanus toxoid (TT) are the average of four or three separate assays, respectively. Other values are the average of duplicate assays. Binding data against insulin and mouse immunoglobulin were similar to those for other irrelevant antigens (not shown). ■, c-*erb*B-2; □, TT; ▨, bovine serum albumin; ▧, heregulin-PE40-FLAG.

Table 1. Deduced amino acid sequence of heavy chain variable region genes of selected anti-c-*erb*B-2 Fabs

	Framework 1	*CDR1*	*Framework 2*	*CDR2*	*Framework 3*	*CDR3*	*Framework 4*
Clone	10 20 30	ab	40	50 ab 60	70 80 abc 90	100cdefgh	103
3A2	SGPGLVKPSQTLSLTCTVSGGSIS	SGGDYWS	WIRQLPGKGLEWIG	YIY--YNGSTYYNPSLKS	RVTLSIDTSKNQFSLKLTSVTAADTAVYYCAR	TGSHFEL-------IP	AANT-VRPLGP
3B3	ESGAEVKKPGASVKVSCKASGYTFI	D--HSIQ	WVRQAPGQGLEWMG	WINP-NSGATNYAERFQD	RVTMTRDTSIATAYMELKSLRSDDTAVYYCAR	SGPTATIIGDSQPLRP	WAQGTLVTVSS
3E2	SGPGLVKPSGTLSLTCAVSGGSIR	ST-NWWN	WVRQPPGKGLDWIG	EIY--LSGYTKYNPSLKS	RVTISIDPSKNQFSLKLTSVTAADTAVYYCAR	DVGTHDYSDSLGAFHI	WGQRTIVTVSS
3E10	SGGNLVQPGGSLRISCSCSGFTFS	V--YALH	WVRQAPGKGLEYIS	GITA-NGGNTYYAHSVEG	RFTISRDNSENTMSLHMSSLRGDDTAVYYCVK	AVKPNWNDRR----RH	WEYWGHGTLIT
3E12	SGPGLVEPSETLSLTCTVSGDSIT	NSLYYWA	WIRQPPGKGLEWIG	IIY--YSRSTYDNPSLMS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	HRPLRHY-------GM	DLWGQGRTVTF
3F2	SGPGLVKPSQTLSLTCTVSGASIS	SGGYHWG	WIRQHPGKGLEWIG	YIY--YRRNTHYNPSLRS	RVIISLDTSKNQFSLKLTSVTAADTAVYYCAR	WGVQLEL-------DY	WGHGTLVTVSS
3H7	SGGGLVRPGGSLRLSCAASGFTFS	H--YEMN	WVRQAPGKGLEWIS	CIIS-GGTTIYYADSAKG	RFTVSRDNAKNTLYLQMNSLRVEDTAIYYCAR	VSQLSTYSLF----DY	WGHGTLITASS
3H11	SGPGLVKPSETLSLTCTVSGGSVS	SGSYYWS	WIRQPPGKGLEWIG	YIY--YTGSTYYNPSLKS	LVTISVDTSKNQFSLKFISVTAADTAVYYCAR	SHYPDKN-------GY	QSHLWGHGSLV
4A2	SGPGLVKPSETLSLTCTVSGGSIS	D--YYWA	WIRQPPGKGLEWIG	YIY--YSGSTNYNSFLRS	RRTISLDTSKNQFSLKLTSVTAADTAVYYCAR	SGAFYGDYYF----DY	WGHGTMVTVSS
4C4	ESGGAVVHPGGSLRLSCAASGFTFT	G--YAMH	WVRQAPGKGLKWVA	LISS-DGNHTYYADSVKG	RFTISRDNSKNTVYLQMNSLRAEDTAVYYCAR	IRGGELRIPYR---DY	WGQGTLVTVSS
4C5	SGPGLLKPSETLSLTCSVSGGSIS	N--SYWS	WIRQSPGKGLEWIG	YIY--SSGSTNYNPSLKS	RLTISVDTSKNQFSLKLTSVTAADTAVYYCAR	SGAFYGDYYF----DY	WGHGTMVTVSS
4D4	SGGGLVQPGGSLRLSCAASGFTFS	S--YEMN	WVRQAPGKGLEWVS	YMSI-DGSSTFYSDSVKG	RFTISRDNAKNSLYLQMNSLRVEDTAVYYCAR	EKWDLLSL------DH	WGQGNLVTVSS

Numbering is according to the V BASE Sequence Directory (Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, UK). Residues in CDR1 and CDR2 differing from germ-line sequences are underlined. CDR, Complementarity determining region; dashes indicate no amino acid in that position in sequence.

 Clinical and Experimental Immunology, **109**:166–174

Table 2. Comparison of heavy chains of FR1, FR2, FR3, CDR1 and CDR2 of human anti-c-*erb*B-2 Fabs showing differences from the most homologous germ-line gene sequence

Clone	3A2	3B3	3E2	3E10	3E12	3F2	3H7	3H11	4A2	4C4	4C5	4D4
V_H family	V_H4	V_H1	V_H4	V_H3	V_H4	V_H4	V_H3	V_H4	V_H4	V_H3	V_H4	V_H3
Germ-line gene	DP-65	DP-75	DP-70	M77301	DP-79	DP-65	DP-58	DP-66	M95144	M77332/Z17396	M95144	DP-58
FR1 nt	2/72 (3%)	4/75 (5%)	2/72 (3%)	6/72 (8%)	4/72 (6%)	1/72 (1%)	1/72 (1%)	1/72 (1%)	0/72 (0%)	5/75 (7%)	2/72 (3%)	0/72 (0%)
FR1 aa	0/24 (0%)	2/25 (8%)	1/24 (4%)	3/24 (13%)	3/24 (13%)	1/24 (4%)	1/24 (4%)	0/24 (0%)	0/24 (0%)	4/25 (16%)	2/24 (8%)	2/24 (8%)
CDR1 nt	1/21 (5%)	6/15 (40%)	2/18 (11%)	3/15 (20%)	6/21 (29%)	2/21 (10%)	2/15 (13%)	1/21 (5%)	4/15 (27%)	1/15 (7%)	2/15 (13%)	0/15 (0%)
CDR1 aa	1/7 (1%)	5/5 (100%)	2/6 (33%)	2/5 (40%)	3/7 (43%)	2/7 (29%)	1/5 (20%)	0/7 (0%)	2/5 (40%)	1/5 (20%)	2/5 (40%)	0/5 (0%)
FR2 nt	1/42 (2%)	2/42 (5%)	0/42 (0%)	2/42 (5%)	1/42 (2%)	1/42 (2%)	2/42 (5%)	0/42 (0%)	0/42 (0%)	6/42 (14%)	2/42 (5%)	1/42 (2%)
FR2 aa	1/14 (7%)	0/14 (0%)	0/14 (0%)	1/14 (7%)	0/14 (0%)	0/14 (0%)	1/14 (7%)	0/14 (0%)	0/14 (0%)	1/14 (7%)	1/14 (7%)	0/14 (0%)
CDR2 nt	4/48 (8%)	7/51 (14%)	6/48 (13%)	11/51 (22%)	7/48 (15%)	6/48 (13%)	5/51 (10%)	2/48 (4%)	3/48 (6%)	7/51 (14%)	2/48 (4%)	10/51 (20%)
CDR2 aa	1/16 (6%)	4/17 (24%)	3/16 (19%)	6/17 (35%)	4/16 (25%)	5/16 (31%)	5/17 (29%)	2/16 (13%)	3/16 (19%)	5/17 (29%)	1/16 (6%)	7/17 (41%)
FR3 nt	8/96 (8%)	9/96 (9%)	7/96 (7%)	6/96 (6%)	4/96 (4%)	7/96 (7%)	5/96 (5%)	9/96 (9%)	7/96 (7%)	2/96 (2%)	5/96 (5%)	2/96 (2%)
FR3 aa	3/32 (9%)	3/32 (9%)	3/32 (9%)	5/32 (16%)	0/32 (0%)	3/32 (9%)	4/32 (13%)	4/32 (13%)	3/32 (9%)	1/32 (3%)	2/32 (6%)	1/32 (3%)

Homologies are based on the V BASE Sequence Directory (Tomlinson *et al*., MRC Centre for Protein Engineering, Cambridge, UK). Percentage differences from germ-line sequences are in parentheses. FR, Framework region; CDR, complementarity determining region; nt, nucleotide; aa, amino acid.

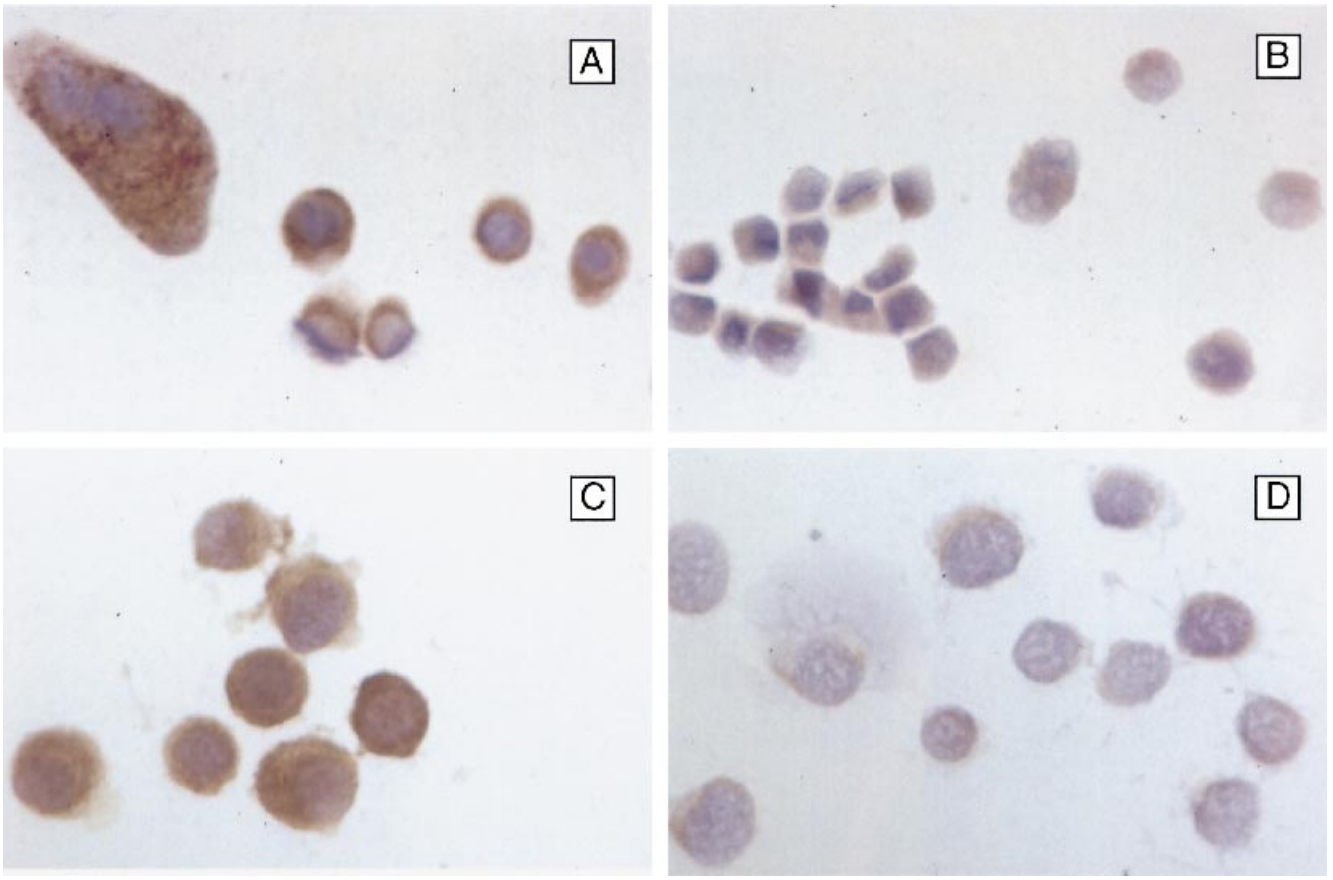

Fig. 2. Immunocytochemical staining of SKBR3 and MDA-MB-231 cells. The Fab 4C5 showed significant staining of the c-*erb*B-2-positive SKBR3 cells (A), but not the c-*erb*B-2-negative MDA-MB-231 cells (B). Fabs 3H11, 4A2, 4C4, 4D4 and 3B3 showed similar patterns of staining. Staining of SKBR3 cells by the Fabs was similar to that seen with the positive control polyclonal rabbit anti-c-*erb*B-2 antibody (C). No staining was observed with the negative control (second and third antibodies only; D).

of clones by Western blotting and ECL. Kappa light chain expression was confirmed in the periplasmic fraction of clones 3B3 and 3H11, and in the bacterial supernatant of clones 4A2, 4C4, 4C5, 4D2, 4D4 and 4F7 by detection of a protein band at ≈ 25 kD in immunoblots.

These Fabs with higher expression levels that had been detected by immunoblot were further assessed for immunoreactivity against the c-*erb*B-2-positive cell line SKBR3 and the negative control cell line MDA-MB-231. Control anti-c-*erb*B-2 bound strongly to SKBR3 cells, but showed no staining of MDA-MB-231 cells above background (Fig. 2C). Five bacterial supernatants (3H11, 4A2, 4C4, 4C5, 4D4) and a periplasmic extract (3B3) showed a similar pattern of staining of SKBR3 cells to that of the control antibody, albeit at a slightly lower level of intensity

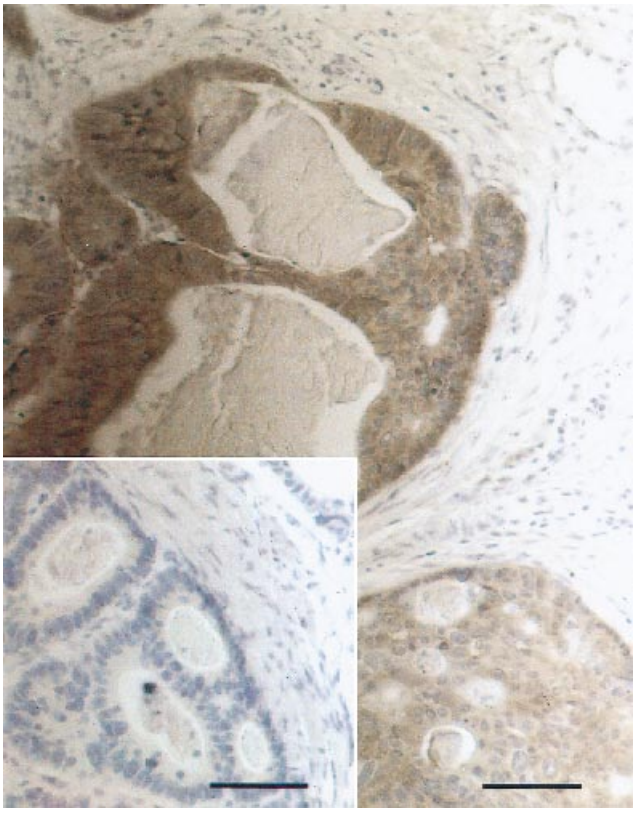

Fig. 3. Immunohistochemical staining of primary colorectal tumour section with polyclonal anti-c-*erb*B-2. Tumour glands show strong cytoplasmic and slight membrane staining with positive control polyclonal rabbit anti-c-*erb*B-2 antibody. Inset shows negative control without primary antibody. Bar represents 100 μm.

(Fig. 2A). These Fabs displayed no significant staining of the MDA-MB-231 cells (Fig. 2B). Remaining Fab samples either failed to show reactivity with SKBR3 (3H11 periplasmic fraction, 3B3 and 4F7 bacterial supernatants) or showed significant cross-reactivity with MDA-MB-231 cells (4D2 supernatant; data not shown).

The primary colonic tumour from the individual from whom the library was constructed was also assessed for c-*erb*B-2 expression. The tumour showed clear evidence of over-expression of the c-*erb*B-2 with considerable cytoplasmic staining of tumour cells and, to a lesser extent, staining of the cell membranes (Fig. 3).

DISCUSSION

We have previously constructed a phage display library from a pericolic lymph node from an individual with colorectal cancer. This study describes the recovery from this library of six unique Fabs with *in vitro* binding specificity for the tumour-associated antigen c-*erb*B-2 on the surface of tumour cells. Antibody fragments with specificity for tumour cells have previously been isolated from individuals immunized with interferon-gamma (IFN-γ)-transduced autologous melanoma cells [21]. Likewise, anti-melanoma scFv fragments have been isolated from cloned B lymphocytes transformed with Epstein–Barr virus (EBV) [22]. However, to our knowledge the present study represents the first report of the isolation by phage display of anti-tumour antibodies constructed with immunoglobulin genes from the lymph node of an individual with cancer.

We sought to gain further information regarding the nature of the immune response in this individual by closer analysis of the isolated Fabs. In undertaking this analysis, it is necessary to recognize that original heavy and light chain pairings may be lost in the process of library construction, and that new antibody specificities may be created. However, there is considerable evidence to suggest that retrieved Fabs nevertheless reflect the changes that are occurring in immunoglobulin gene usage *in vivo*. First, antibody phage display has been successful in the isolation of specific antibodies from libraries constructed with lymphocytes from immune hosts [16,21,23], even where those libraries were of such a size ($\approx 10^7$ clones) that retrieval of original pairings was unlikely. It is also clear that a considerable proportion of the antigenic specificity of Fabs is conferred by the heavy chain, and that this specificity can be maintained despite pairing with a number of different light chains—the concept of chain promiscuity [24,25]. Finally, recent studies have provided direct evidence for the contention that Fab fragments isolated from combinatorial libraries reflect those heavy and light chain combinations commonly found in the *in vivo* repertoire [26,27]. Thus while original pairings will not necessarily be maintained, it is likely that the individual antibody genes of the cloned Fabs will represent those used by reactive lymphocytes present in the original lymph node tissue.

A further consideration in the analysis of isolated Fabs is the limitation in diversity of immunoglobulin chains introduced by the use of an IgG1/K library, rather than libraries containing all possible heavy and light chain genes. However, since the purpose of this study was to identify and analyse those anti-c-*erb*B-2 antibodies present in the enriched library, rather than all or even most of those present in the individual, we consider that the use of this library was appropriate.

Anti-tumour antibodies may represent either a specific immune response to tumour-associated antigens, or alternatively, a non-specific response arising independent of antigenic challenge. Antibodies resulting from the latter response are typically of low affinity and polyreactive, in that they recognize a variety of self and non-self antigens [28]. Furthermore, they generally demonstrate limited somatic mutation and show preferential usage of certain germ-line variable region genes [29]. We were able to identify 16 unique clones which were reactive with c-*erb*-B2 but which showed no binding to a panel of irrelevant antigens, including some antigens known to be targets for polyreactive antibodies (IgG, TT) [28]. From our results, we estimate that Fabs with polyreactivity represented <1% of all clones isolated by panning. It is possible that polyreactive antibodies against c-*erb*B-2 were present in the individual but were selected against during the process of panning. The construction of the library from γ_1 heavy chains may also have biased the library against polyreactive antibodies, since these are frequently of the IgM isotype [28].

Sequence analysis was perfomed on V_H rather than V_L gene segments, since they show greater rates of somatic mutation, and are usually the major determinant of antigen complementarity. Given the probable loss of original pairings and resultant light chain promiscuity, it was considered that the heavy chains were more likely to reflect antigen-driven events occurring *in vivo*. We found considerable variation in the V_H regions of the Fabs examined by nucleotide sequencing with three different heavy chain families being represented. In terms of functional germ-line V_H gene segments, V_H3 is known to be the most abundant, followed by V_H4 and V_H1 [30]. While limited data are available on their usage by lymphocytes involved in specific immune responses, this preponderance of V_H3 gene usage appears to be maintained. In an analysis of V_H genes used by hybridoma-derived antibodies, 47% were V_H3, 35% were V_H1, and 13% were V_H4 [25]. Antibodies isolated using phage display show a similar profile of V_H gene usage, irrespective of whether they reacted with self-antigens [31] or exogenous protein antigens [25]. All of the anti-c-*erb*B-2 Fabs isolated in this study used commonly represented V_H genes (V_H4, V_H3, V_H1). The apparent over-representation of V_H4 is of some interest, although its interpretation is difficult in view of the small numbers of clones involved. Importantly, we do not feel that the V_H4 predominance reflects a bias introduced in library construction, as we have previously used the same library to retrieve Fabs against TT that were predominantly V_H3 [7]. The CDR1 and CDR2 regions of most clones showed considerable divergence from the most homologous germ-line V_H gene, indicating that each gene had undergone significant somatic mutation.

Taken together, we consider the findings of antigen specificity, V_H gene usage and somatic mutation provide strong support for the contention that the isolated Fabs were constructed from immunoglobulin genes fashioned by a specific humoral immune response to c-*erb*B-2. This hypothesis is further supported by the finding that c-*erb*B-2 was over-expressed on the original tumour. While c-*erb*B-2 is a non-mutated self protein, the presence of serum antibodies has been associated with both mutated and over-expressed oncogenes by tumour cells [14]. Recent work has shown that cytoplasmic staining of c-*erb*B-2 is present in $\approx$20% of primary colorectal tumours and is associated with the presence of serum antibodies (Ward *et al.*, unpublished). Although serum from the patient was not available for analysis, over-expression of c-*erb*B-2 on the primary tumour supports the hypothesis that the

isolation of these recombinant Fabs was the indirect result of a specific immune response to the tumour.

A single-chain Fv antibody against the extracellular domain of c-*erb*B-2 has previously been isolated by panning a combinatorial antibody library [19,32] constructed from peripheral blood mononuclear cells from a healthy individual. Clearly, antibodies isolated through this process are unlikely to reflect a naturally occurring immune response. In contrast, our experimental approach was designed to maximize the chance of isolating the genes of such naturally occurring antibodies. The library was constructed from a pericolic lymph node which drained the tumour but was not involved by metastatic disease. We considered that nodal tissue was the most likely site for the occurrence of a humoral immune response to tumour antigens, and indeed such tissue has been used to isolate anti-thyroid antibodies from an individual with autoimmune thyroiditis [33]. Furthermore, the c-*erb*B-2 used for these experiments was expressed in mammalian cells, since the resultant glycosylated proteins would serve as a better target for the isolation of naturally occurring antibodies.

We see several potential benefits in the exploitation of existing immune responses for the isolation of recombinant human antibodies. Natural selection of immunoglobulin genes in the presence of antigen has the potential to produce antibodies of greater specificity and affinity than those seen in all but the largest synthetic or non-immune libraries. At the same time, this approach may limit the presence in libraries of genes encoding antibodies with specificity for self antigens or antibodies with inherent immunogenicity. In a broader context, the isolation of anti-c-*erb*B-2 Fabs from an immune library, rather than from a semisynthetic or non-immune library, may facilitate examination of the antigen epitopes recognized *in vivo*, thereby increasing our understanding of the immune response to tumour cells.

In conclusion, our results indicate that a naturally occurring immune response to disease-related antigens can be exploited to provide source material for combinatorial phage display libraries of moderate size. By using the recombination and maturation events of the *in vivo* humoral immune response, these libraries appear to constitute an enriched source of relevant immunoglobulin genes. Thus, they may provide a convenient and appropriate source for the isolation of immunoglobulin genes encoding Fabs to predefined target antigens.

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REFERENCES

1 Barbas C III, Rosenblum JS, Lerner RA. Direct selection of antibodies that coordinate metals from semisynthetic combinatorial libraries. Proc Natl Acad Sci USA 1993; **90**:6385–9.

2 Williamson RA, Burioni R, Sanna PP *et al.* Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. Proc Natl Acad Sci USA 1993; **90**:4141–5.

3 Griffiths AD, Williams SC, Hartley O *et al.* Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 1994; **13**:3245–60.

4 Nissim A, Hoogenboom HR, Tomlinson IM *et al.* Antibody fragments from a 'single pot' phage display library as immunochemical reagents. EMBO J 1994; **13**:692–8.

5 Vaughan TJ, Williams AJ, Pritchard K *et al.* Human antibodies with sub-nanomolar affinites isolated from a large non-immunized phage display library. Nature Biotechnol 1996; **14**:309–14.

6 Griffiths AD, Malmqvist M, Marks JD *et al.* Human anti-self antibodies with high specificity from phage display libraries. EMBO J 1993; **12**:725–34.

7 Ward RL, Clark MA, Lees J *et al.* Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. J Immunol Methods 1996; **189**:73–82.

8 Soderlind E, Vergeles M, Borrebaeck C. Domain libraries— synthetic diversity for de novo design of antibody v-regions. Gene 1995; **160**:269–72.

9 Geoffroy F, Sodoyer R, Aujame L. A new phage display system to construct multicombinatorial libraries of very large antibody repertoires. Gene 1994; **151**:109–13.

10 Waterhouse P, Griffiths AD, Johnson KS *et al.* Combinatorial infection and *in vivo* recombination: a strategy for making large phage antibody repertoires. Nucl Acids Res 1993; **21**:2265–6.

11 Cha S, Leung PSC, Gershwin ME *et al.* Combinatorial autoantibodies to dihydrolipoamide acetyltransferase, the major autoantigen of primary biliary cirrhosis. Proc Natl Acad Sci USA 1993; **90**:2527–31.

12 Schlichtholz B, Legros Y, Gillet D *et al.* The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. Cancer Res 1992; **52**:6380–4.

13 Labrecque S, Naor N, Thomson D, Matlashewski G. Analysis of the anti-p53 antibody response in cancer patients. Cancer Res 1993; **53**:3468–71.

14 Disis ML, Calenoff E, McLaughlin G *et al.* Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res 1994; **54**:16–20.

15 Pupa SM, Menard S, Andreola S, Colnaghi MI. Antibody response against the c*erb*B-2 oncoprotein in breast carcinoma patients. Cancer Res 1993; **53**:5864–6.

16 Zebedee SL, Barbas C3, Hom YL *et al.* Human combinatorial antibody libraries to hepatitis B surface antigen. Proc Natl Acad Sci USA 1992; **89**:3175–9.

17 Evan GI, Lewis GK, Ramsay G *et al.* Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol Cellular Biol 1985; **5**:3610–6.

18 Fiddes RJ, Janes PW, Sanderson GM *et a.l* Heregulin (HRG)-induced mitogenic signaling and cytotoxic activity of a HRG/PE40 ligand toxin in human breast cancer cells. Cell Growth Different 1995; **6**:1567–77.

19 Marks JD, Hoogenboom HR, Bonnert TP *et al.* By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 1991; **222**:581–97.

20 Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest, 5th edn. Bethesda: US Dept of Health and Human Services, Public Health Service, National Institutes of Health, 1991.

21 Cai XH, Garen A. Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells—selection of specific antibodies from single-chain fv fusion phage libraries. Proc Natl Acad Sci USA 1995; **92**:6537–41.

22 Zhang H, Lake DF, Barbuto J *et al.* A human monoclonal antimelanoma single-chain fv antibody derived from tumor-infiltrating lymphocytes. Cancer Res 1995; **55**:3584–91.

23 Griffin HM, Ouwehand WH. A human monoclonal antibody specific for the leucine-33 (PI^{A1}, HPA-1a) form of platelet glycoprotein IIIa from av gene phage display library. Blood 1995; **86**:4430–6.

24 Collet TA, Roben P, O'Kennedy R *et al.* A binary plasmid system for shuffling combinatorial antibody libraries. Proc Natl Acad Sci USA 1992; **89**:10026–30.

25 Ohlin M, Borrebaeck CAK. Characteristics of human antibody repertoires following active immune responses *in vivo*. Mol Immunol 1996; **33**:583–92.

26 Caton AJ, Koprowski H. Influenza virus hemagglutinin-specific antibodies isolated from a combinatorial expression library are closely related to the immune response of the donor. Proc Natl Acad Sci USA 1990; **87**:6450–4.
27 Reason DC, Wagner T, Lucas AH. Human Fab fragments specific for the *Haemophilus influenzae* b polysaccaride isolated from a bacteriophage combinatorial library use variable region gene combinations and express an idiotype that mirrors *in vivo* expression. Infect Immun 1997; **65**:261–6.
28 Casali P, Notkins AL. $CD5^+$ B lymphocytes, polyreactive antibodies and the human B cell repertoire. Immunol Today 1989; **10**:364–8.
29 Ditzel HJ, Barbas SM, Barbas C III *et al.* The nature of the autoimmune antibody repertoire in human immunodeficiency virus type 1 infection. Proc Natl Acad Sci USA 1994; **91**:3710–4.
30 Cook GP, Tomlinson IM. The human immunoglobulin VH repertoire. Immunol Today 1995; **16**:237–42.
31 Rapoport B, Portolano S, McLachlan SM. Combinatorial libraries: new insights into human organ-specific autoantibodies. Immunol Today 1995; **16**:43–49.
32 Schier R, Bye J, Apell G *et al.* Isolation of high-affinity monomeric human anti-c-*erb*B-2 single chain fv using affinity-driven selection. J Mol Biol 1996; **255**:28–43.
33 Mcintosh RS, Asghar MS, Watson PF, Kemp EH, Weetman AP. Cloning and analysis of IgG-kappa and IgG-lambda anti-thyroglobulin autoantibodies from a patient with Hashimoto's thyroiditis—evidence for *in vivo* antigen-driven repertoire selection. J Immunol 1996; **157**:927–35.