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Identification of colon tumour-associated antigens by phage antibody selections on primary colorectal carcinoma

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Abstract

Immunotargeting of solid tumours using antibodies has become a valuable tool for the detection of cancer metastases and the treatment of minimal residual disease. However, only few tumour antigens useful for targeting have been described to date. To identify cell-surface targets on colorectal carcinoma (CRC), we selected a large, human phage antibody repertoire on freshly isolated colon tumour cells. Two antibodies were identified that reacted with epithelial cell-restricted cell-surface antigens, whereas one clone preferentially reacted with stromal cells. These antigens are tumour-associated antigens, as shown by their uniform expression in tumours of different patients and of different differentiation stages and by their limited expression on normal tissues. The pattern of reactivity in immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) suggests that these antigens are different from previously identified tumour-associated antigens (e.g. Ep-CAM or c-ERB-2). This phage antibody-based method may lead to the cloning of novel tumour antigens that are useful for the immunotargeting of solid tumours.

Keywords: Colon cancer; Phage display; Tumour antigen; Cell selection

1. Introduction

Colorectal cancer (CRC) is the second most prevalent cancer in the western world. The principal treatment modality is surgical removal of the primary tumour and adjuvant chemotherapy (at present, 6 months of 5-fluorouracil (5-FU) plus leucovorin (folinic acid) can be considered standard therapy) for metastatic disease. However, especially for minimal residual disease, immunotherapy using monoclonal antibodies directed to tumour-associated antigens has proven to be a promising new treatment modality [1,2]. However, most of the target antigens described to date for colon cancer (e.g. carcinoembryonic antigen (CEA), c-ERB-2, TAG72, Ep-CAM) are tumour-associated and thus are also expressed on normal colon. This may give rise to toxicity problems when these antigens are used as targets in the treatment of patients [3].

The search for new target antigens expressed on cancer cells has traditionally involved the immunisation of mice with cancer cells, cell lines or cell-extracts and the subsequent screening of hybridomas for antibodies that bind to the target cell [4]. In addition, tumour antigens have been identified by the cloning of T-cells that recognise major histocompatibility class I (MHC-I)-restricted peptides derived from the antigen [5]. More recently, genetic approaches are increasingly being used to define genes that are associated with malignant transformation [6] or to define distinct genetic subtypes of malignancies [7] at the transcriptional level.

Antibody phage display (reviewed in [8]) has also been used as a means to quickly select antibodies specific for certain cell types or tissues [9,10] or known tumour antigens [11,12]. This strategy may be used to identify cell-surface structures on cancer cells: novel antigens can then be defined by means of the antibodies that recognise them. Although this approach has already

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been successfully applied to melanoma [13] and also, to a lesser extent, to lung carcinoma [14], until now, only one study has been reported that describes the selection of antibodies reactive with CRC antigens [15].

The success of this approach depends on many parameters, including the phage antibody library size and quality and particularly on the selection method used. We have previously described a study of phage antibody selections on complex antigens, such as cell surfaces, in which we investigated the parameters important for successful selection [16]. These models were used to define selection criteria for efficient selection on whole tumour cells. Here, we describe the use of these protocols for the selection of human antibody fragments reactive with colon cancer. By direct panning of a large, non-immunised phage antibody repertoire [17] on freshly isolated colon tumour cells, we hoped to retrieve antibodies that recognise patient-derived tumour material in immunohistochemistry (IHC) analysis.

2. Materials and methods

2.1. Escherichia coli *strain*

TG1: K12, Δ*(lac-pro)*, *supE*, *thi*, *hsdD5*/F'*traD*36, *pro*A^+B^+, *lacI*^{q}, *lacZ*ΔM15

2.2. Cell line

The colorectal cancer cell line CaCo2 was obtained from the American Type Culture Collection (ATCC; number HTB37) and was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Phage antibody selection on primary colorectal carcinoma

A large, fully human, non-immunised phage antibody repertoire [17] was used for selection. The library was rescued with helper phage M13K07 and phages were purified and concentrated from the bacterial culture supernatant by polyethylene glycol (PEG) precipitation as described in [18]. After each selection round, the percentage of bacterial clones containing complete (Fab) insert was also determined, since a loss of clones containing V-gene inserts is representative of inefficient selection.

After surgery, primary colorectal cancer tissue was obtained from the department of Pathology. Tissue was washed three times with 20 ml of phosphate-buffered saline (PBS) (50 mM phosphate buffer (KH_2PO_4/Na_2HPO_4: pH 7.4); 150 mM NaCl), cut in small pieces using a sterile scalpel blade and washed again with PBS. Tissue pieces were allowed to settle and buffer supernatant was removed. Cells were detached by overnight incubation at 4°C in isolation buffer (PBS, containing 1 mM EDTA, 1 mM EGTA and 0.5 mM dithiothreitol (DTT) with gentle shaking. Next day, cells were harvested by centrifugation (100*g*, 3 min, 4°C), washed three times with 10 ml of PBS by centrifugation and resuspension and fixed in 5 ml 0.25% (w/v) paraformaldehyde in PBS for 20 min at 4°C. Cells were washed three times again with 10 ml of PBS, pelleted (100*g*, 3 min, 4°C) and resuspended in 1 ml of PBS. Phages were blocked for 1 h at room temperature in 1 ml of 4% (w/v) Marvell (skimmed milk powder) in PBS (4% MPBS), added to the cell suspension and incubated for $1\frac{1}{2}$ h at room temperature with gentle shaking. Cells were washed five times with 5 ml of PBS by centrifugation and resuspension and phages were eluted with 1 ml of 100 mM tri-ethylamine (TEA; pH = 12) for 10 min at room temperature. The eluate was neutralised with 0.5 ml 1 M Tris-Cl (pH 7.5) and phages were allowed to infect exponentially growing *E. coli* TG1 and titrated as described in [18].

2.4. Characterisation of selected antibody fragments by DNA fingerprinting and immunohistochemistry

After selection, antibody-encoding V-genes of selected antibody fragments were amplified by polymerase chain reaction (PCR) from a single bacterial colony using primers pUC-reverse (5′-AGC GGA TAA CAA TTT CAC ACA GG-3′) and fd-TET seq (5′-TTT GTC GTC TTT CCA GAC GTT AGT-3′). The percentage of bacterial clones containing complete (Fab) inserts was then determined, since a decrease of clones containing V-gene insert is representative of inefficient selection. Finally, antibody V-genes were DNA-fingerprinted as described in [18].

For immunohistochemistry (IHC), 5 micrometre cryosections of different cancerous and normal tissues were cut and mounted on Starfrost Adhesiv (Klinipath, Duiven, The Netherlands) glass slides. Slides were dried overnight at room temperature and stained with recombinant Fab antibody fragments essentially as described for single-chain Fv (scFv) by Roovers and colleagues [19]. However, slides were fixed with 1% (w/v) paraformaldehyde in PBS for 20 min at room temperature (instead of with acetone) before staining with recombinant antibody fragments. Large scale production and purification of antibodies that were reactive in IHC using immobilised metal ion affinity chromatography (IMAC) and fast protein liquid chromatography (FPLC) were as described for scFv by Roovers and colleagues [19]. Fab fragments were purified via their c-terminal hexahistidine sequence and detected by

means of their *c-myc*-derived epitope tag, both encoded in the phagemid vector pCES1 [17].

2.5. Analysis of cell surface-binding of selected antibody fragments by means of flow cytometry

Adherently growing CaCo2 cells were harvested at subconfluency by trypsin/EDTA treatment and washed once with PBS. Cells were either used directly, or fixed with 1% (w/v) paraformaldehyde in PBS for 20 min at room temperature. Subsequently, cells were washed twice with 2% (w/v) bovine serum albumin (BSA) in PBS and aliquots of approximately 5×10^5 cells were made. All incubations were carried out for 1 h at room temperature in either 2% MPBS containing 0.05% (w/v) NaN_3 (2% MPBS/N_3) for non-fixed cells, or in 2% MPBS for paraformaldehyde-fixed cells. Cells were washed twice with 0.5 ml of the respective buffer between every incubation step. Staining was performed with purified, recombinant antibody fragments (approximately 100 nM), the 9E10 antibody ([20]; 50% (v/v) hybridoma supernatant in 4% MPBS) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark; 2% (v/v)). Cells were finally resuspended in 0.5 ml of PBS and staining was visualised by flow cytometry using a fluorescent activated cell sorter (FACS)-Calibur (Becton & Dickinson, Heidelberg, Germany).

3. Results

To identify possibly novel cell-surface antigens on CRC, we set out to select phage antibodies with specificity for colon tumour cells by panning a very large Fab phage antibody repertoire [17] against freshly isolated tumour cells. For practical reasons, a new tumour-cell isolation from a tumour of a different patient was performed for every round of selection; therefore, the amount of cells differed between selections. Table 1

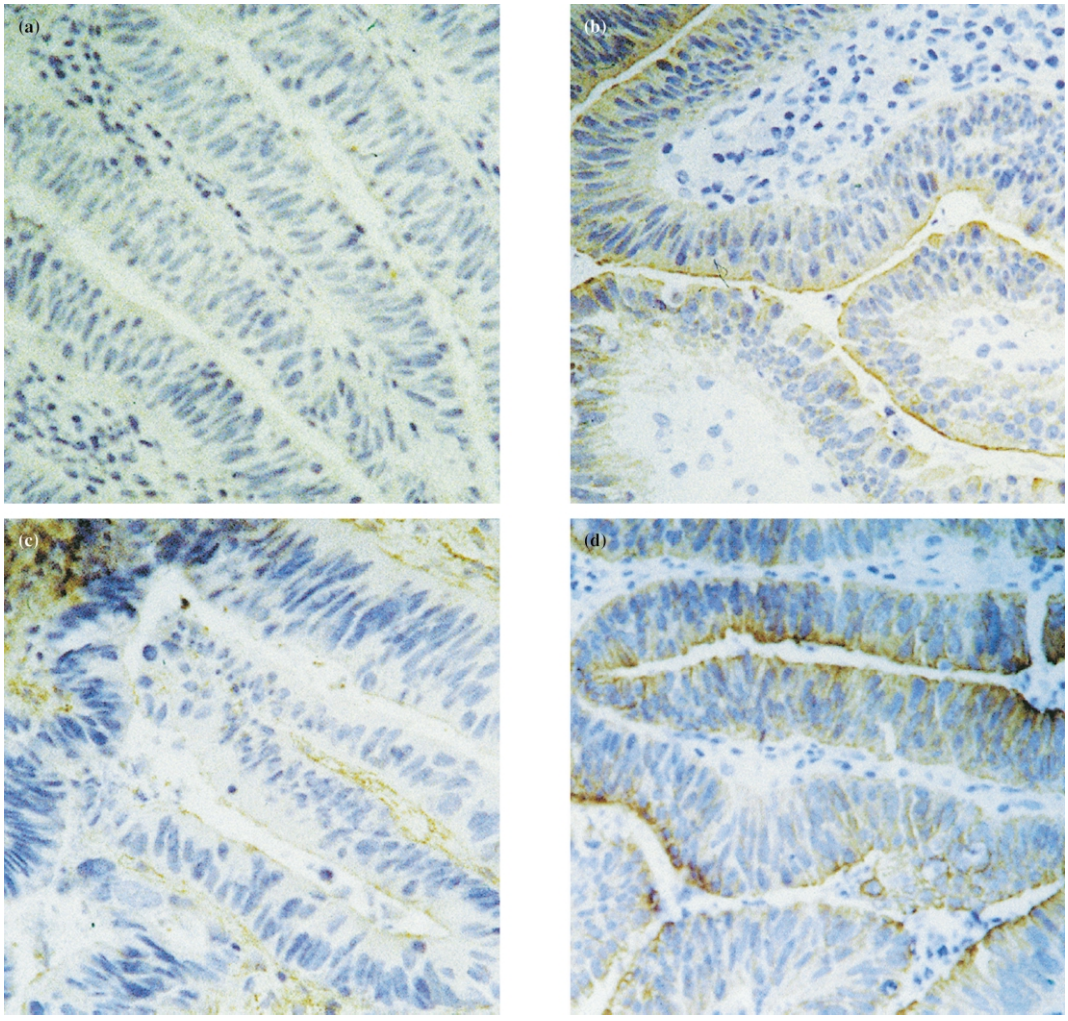

Fig. 1. Immunohistochemical staining of tissue cryosections of colon carcinoma using recombinant Fab antibody fragments. Bound antibody fragments were detected with the 9E10 antibody recognising a C-myc-derived epitope tag and peroxidase-labelled antimouse antibodies: (a) negative control (a Fab antibody directed to tetanus toxoid); (b) clone B5; (c) clone B8; (d) clone C8.

Table 1
Characteristics of phage antibody selections and percentage of clones containing full-length insert

Selection round	Input titre[a] (i)	Output titre[a] (o)	Ratio o/i	Enrichment factor[b]	% full-length clones
1	4.2×10^{12}	2.6×10^{7}	6.2×10^{-6}	n.a.	n.d.
2	2.4×10^{13}	5.0×10^{7}	2.1×10^{-6}	0.34	n.d.
3	1.8×10^{13}	3.3×10^{8}	1.8×10^{-5}	8.57	83
4	8.0×10^{12}	4.9×10^{7}	6.1×10^{-6}	0.34	94
5	2.5×10^{13}	4.1×10^{8}	1.6×10^{-5}	2.62	100

n.a., not applicable; n.d., not determined.
[a] Titres are given as colony-forming unit (cfu)/ml.
[b] Enrichment factor is defined as: (Ratio o/i selection round $n+1$)/(Ratio o/i selection round n).

shows the results of selections: there was no clear increase in phage titre, but this may be explained by the differences in the number of cells used for selection. However, the percentage of clones containing full-length Fab insert remained high and even increased slightly with every selection round, hinting towards successful selection (Table 1). After four and five rounds of selection, the diversity of the selected phage antibody population was examined and clones having a distinct fingerprint pattern (25 after round four and 17 after round five) were subsequently tested for their reactivity towards CRC tissue in immunohistochemistry (IHC).

Table 2
Overview of V-, D- and J-segment usage of selected phage antibodies

V-gene	V-gene family	Closest germ line V-gene segment	D-segment used	J-segment used
VH (clone B5)	VH3	DP-77/WGH16+	D5-5	JH4b
VL (clone B5)	Vκ3	Vg/38k	n.a.	Jκ2
VH (clone B8)	VH3	VHGL3.7/DP-82	D6-25	JH6b
VL (clone B8)	Vκ2	DPκ27/A29+	n.a.	n.d.
VH (clone C8)	VH3	cos-8/hv3005f3	D6-13/DN1	JH4b
VL (clone C8)	Vλ3	DPL16/VL3.1	n.a.	JL2/JL3a

n.a., not applicable. n.d., using the sequence alignment software of V-base: (http://www.mrc-cpe.cam.ac.uk/imt-doc/restricted/DNA-PLOT.html), the segment could not be determined.

Table 3
Expression profile of antigens recognised by selected phage antibody fragments on a panel of normal tissues

Tissue	Staining by Fab B5	Staining by Fab B8	Staining by Fab C8
Breast	Membranous and cytoplasmic + on epithelial cells	n.d.	–
Colon	Membranous + on epithelial cells	Smooth muscle cells and endothelial cells slightly +	Membranous + on epithelial cells
Ileum	Mostly membranous+ on epithelial cells only	–	Membranous and cytoplasmic+ on epithelial cells only
Kidney	Only distal tubuli + glomeruli –	–	Proximal/distal tubuli + glomeruli –
Larynx	–	–	–
Liver	Only bile ducts slightly+hepatocytes –	–	Bile ducts and hepatocytes +
Lung	Bronchial, cubic epithelium +	–	Bronchial, cubic epithelium +
Lymph node	–	–	–
Muscle	–	Endothelial cells slightly +	–
Ovarium	–	–	Inclusion-cysts +
Pancreas	Exocrine part + endocrine part –	n.d.	Exocrine part + endocrine part –
Skin	–	–	–
Spleen	–	–	–
Stomach: antrum	Membranous and cytoplasmic + on epithelial cells	Very locally + mostly –	Membranous and cytoplasmic + on epithelial cells
Thyroid	(Focally) very slightly +	Some smooth muscle cells slightly +	Strongly membranous+with apical accentuation
Uterus	–	–	Epithelium of the basal parts of the tubules +

+, postitive; –, negative.

After four rounds of phage selection, no positive signals were observed in the screening of different selected antibodies by means of IHC using colon tumour tissue of a different patient. However, after five rounds of selection, three out of 17 different *BstNI* fingerprint patterns tested, scored positive in IHC (Fig. 1). Two of the antibodies exclusively stained malignant epithelial cells (clones B5 and C8): the observed staining pattern was mostly membranous, but also partially cytoplasmic (Fig. 1b and d). The remaining clone (B8) reacted very slightly with epithelial cells, but strongly stained stromal cells (e.g. fibroblasts; Fig. 1c). The V-genes of these selected antibodies were sequenced and shown to originate from different, but commonly used V-gene families (Table 2). *VH* genes were all derived from the largest (VH3) gene family and light chain genes (*VL*) originated from both (κ and λ) isotypes (Vκ2 and 3 and Vλ3 families; Table 2). Production of the antibodies as soluble Fab fragments in *E. coli* revealed that all antibodies were expressed as disulphide-bridged heterodimers of light and heavy chains of approximately 45 kDa. All antibodies could be purified to more than 90% by one-step IMAC (Fig. 2a). In addition, FPLC analysis of IMAC-purified Fab showed a single peak at the retention time expected for a protein of approximately 50 kDa (Fig. 2b). The yield of purified antibody fragment varied between 600 and 800 μg per l of bacterial culture.

To validate the antigens recognised by the selected antibody fragments as putative colon tumour antigens, their presence on several different colon tumours ($n = 10$) of various differentiation (Dukes' A–C) was tested by IHC analysis. All three antigens were expressed in all tumours tested, giving the same staining pattern as shown in Fig. 1. To examine whether the target antigens were also expressed in different adenocarcinomas than CRC, staining of carcinomas of the stomach, lung and breast was performed. Clone C8 showed strong homogeneous positivity for all tumours; Fab B5 showed strong positivity for breast and lung carcinoma, but was more heterogeneously reactive with stomach carcinoma and clone B8 produced negative stains for all these tumours. To characterise the antigens further, their expression profile on a panel of different normal tissues was determined by IHC (Table 3). Clones B5 and C8 recognise antigens expressed in several normal epithelia: both B5 and C8 react with various tissues of the gastrointestinal tract (antrum, ileum and colon) and lung tissue. However, marked differences between the clones B5 and C8 were also observed: whereas antibody C8 strongly stained follicular epithelial cells in the thyroid, hepatocytes in liver and proximal and distal tubuli in kidney, clone B5 showed little staining of the thyroid or liver and only stained distal tubuli in the kidney (Table 3). Furthermore, Fab C8 did not react with breast epithelium, whereas clone B5 showed positive staining of this tissue. Finally, Fab C8 bound to the epithelium of the basal parts of the tubules in the uterus, whereas Fab B5 did not. Antibody B8 showed only very weak reactivity towards endothelial cells in muscle and colon and to smooth muscle cells in colon and thyroid, but showed mostly negative staining of the tissues tested (Table 3).

In a first experiment to determine the molecular weight of the target antigens, purified, recombinant Fab antibody fragments were used in immunoblotting. Total cellular protein extracted from the CRC cell line CaCo2 was blotted to nitrocellulose and then probed with purified Fab. None of the three selected antibody fragments recognised their cognate antigen in this test, meaning that they might recognise non-linear epitopes that are sensitive to denaturation of the antigen (data not shown). In addition, immunoprecipitation using capture of native antigen in solution did not result in the precipitation of a specific protein (data not shown). These

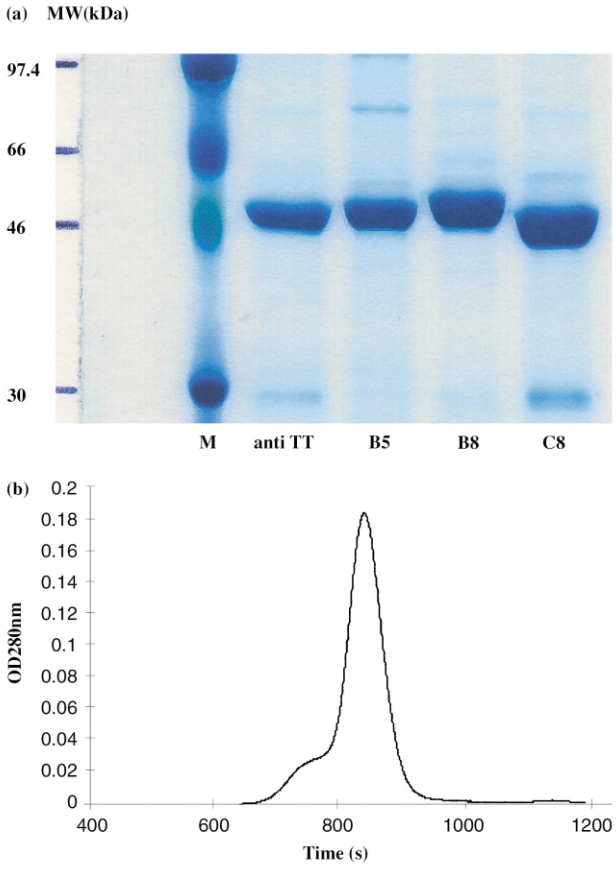

Fig. 2. Purification of selected, recombinant antibody fragments. Antibody fragments were produced in *E. coli* and purified from the periplasmic space by means of immobilised metal ion affinity chromatography (IMAC): (a) sodium duodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of IMAC-purified Fab; proteins were visualised by means of Coomassie Brillant Blue staining; (b) fast protein liquid chromatography (FPLC) analysis of IMAC-purified Fab; absorbance (OD 280 nm) of the flow-through of the column was measured as a function of the retention time. M, marker; anti-TT, antitetanus toxoid; MW, molecular weight.

results show that the epitopes recognised by the antibody fragments could well be dependent on chemical modification of the antigen expressed on the cell-surface. Indeed, flow cytometric analysis of CaCo2 cells using Fab fragments B5 and C8 showed that antibody reactivity was abolished when cells were not fixed with paraformaldehyde (Fig. 3) or treated with a reducing agent (DTT; data not shown). However, both antibody fragments did strongly stain paraformaldehyde-fixed whole CaCo2 cells, proving that the antigens recognised by the antibodies B5 and C8 are cell-surface markers.

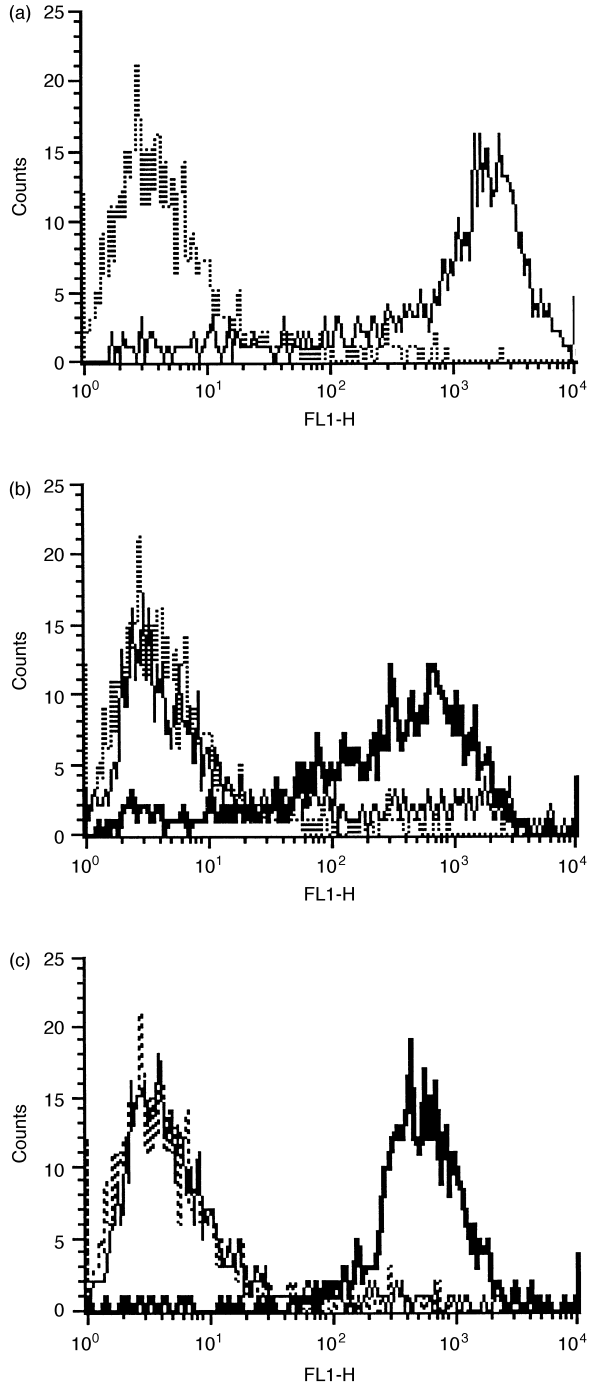

Fig. 3. Cell-surface binding of purified, recombinant antibody fragments B5 and C8 as demonstrated by flow cytometric analysis using the CRC cell line CaCo2. Histogram plots showing the number of gated whole cells (counts) as a function of their fluorescence intensity (FL1-H). Cells were either non-fixed, or fixed with 1% (w/v) paraformaldehyde prior to staining. Negative control staining (an antibody to the antigen tetanus toxoid) is shown in all panels by a dotted line: (a) the anti Ep-CAM antibody MOC-31 (thin line) as a positive control; (b) Fab B5 staining of untreated cells (thin line) and fixed cells (thick line); (c) Fab C8 staining of untreated cells (thin line) and fixed cells (thick line).

4. Discussion

In this report, we describe the successful isolation of antibodies with specificity for antigens expressed in colorectal carcinoma by panning a large, non-immunised phage antibody library on colon tumour cells isolated from patient-derived tissue. Antibodies B5 and C8 recognise epithelial cell-specific antigens and antibody B8 preferentially reacts with stromal cells in colon tumours only. These antigens are of interest, because they are expressed in sections of all ($n=10$) colon tumours tested and in only a subset of normal epithelial tissues. They are not tumour-specific in the strict sense, but may be classified as tumour-associated antigens. Future quantitative expression studies are needed to further validate these antigens as tumour markers.

The selection protocol used for phage antibody selection was designed to direct the selection towards epitopes common to different tumours and to avoid possible selection of antibodies directed to antigens unique for one, or a subset of patients (e.g. human leucocyte antigen (HLA) molecules). Therefore, patient-derived tumour material was chosen as an antigen source, instead of a tumour cell line and tumours used were not matched or selected for any stage or differentiation. Finally, the screening of selected antibody fragments was performed on sections of a randomly picked, allogeneic tumour. Indeed, the three phage antibodies selected showed reactivity towards CRC tissue of all the different patients tested ($n=10$). However, the abundance of clones that did not react in IHC (14 out of 17 different antibodies tested after five rounds of selection) may indicate that numerous antibodies specific for a subset of patients were selected. Alternatively, the expression levels of these Fabs or of their cognate antigens may have been too low to permit successful staining in IHC. Since the selected pool of antibodies after five selection rounds was still diverse and only a limited number of the selected clones were tested, it is expected that many more specificities may be identified by additional screening.

Selections were performed on patient-derived tumour material which was treated with DTT to extract tumour cells from the surrounding extracellular matrix and tumour cells were fixed with paraformaldehyde to preserve the cell surface during the selection procedure.

Therefore, it is no surprise that the epitope recognised by all three selected antibody fragments depends on modification of the cell surface. These antibody fragments themselves are therefore not useful for *in vivo* targeting of CRC, but may be of value for immunodiagnosis of CRC. In this respect it is noteworthy that only Fab C8 (and not B5 or B8) recognises its cognate antigen in formalin-fixed, paraffin-embedded tissue sections, which may enlarge its usefulness for diagnostic applications (data not shown).

The use of a direct selection strategy without depletion or subtraction does not avoid the retrieval of antibodies that cross-react with normal tissue, as witnessed by the reactivity of the selected clones towards a subset of normal tissues (Table 3). To direct the selection towards tumour-specific epitopes, depletion and/or subtraction strategies should be used [9,15,21]. In addition, future experiments should be designed such that the selection of antibodies reactive with modified antigens is avoided. A number of different selection strategies may be envisioned, including selections on labelled cell lysates or membrane preparations, or the use of tissue sections. Phage antibody selections on tissue cryosections of patient material have already been successfully used [22]. Furthermore, in the model system we described in [16], we also have found that moderate enrichment factors of specific phage can be achieved using this source of antigen. Therefore, we have also explored this selection strategy for the selection of antibodies to colon tumour antigens. However, after multiple rounds of selection, a loss of phage clones containing Fab inserts was observed, indicating that the selection pressure was not sufficient for the selection of binding phages (data not shown). This was probably caused by the limited amount of antigen present in tissue cryosections.

Expression of tumour-associated antigens in normal tissue may cause toxicity in patients when the antigen is used as target for immunotherapy [3]. However, for radio-immunodiagnosis, low-level expression in normal tissue may be less of a problem [23,24]. In the latter setting, less antibody is given to patients causing less toxicity problems in antigen-positive normal tissues, even when the antigen is shed into the circulation [25]. The normal tissues that are important for dose-limiting toxicity are blood cells when radio-immuno targeting (RAIT) is used [26] and mainly the liver and kidney when unconjugated antibody is used. The antigen recognised by selected antibody B5 is not present on hepatocytes and only on the distal tubuli in the kidney, thereby possibly avoiding an important cause of toxicity in patients. However, accumulation of antibody fragments in the kidney will mostly depend on the antibody format (i.e. size and charge) used for targeting and will therefore be largely antigen-independent. Finally, none of the target antigens were expressed on lymphocytes, as witnessed by the absence of staining in lymph node tissue. This shows that, although the antibodies selected in this study are unsuitable for *in vivo* use, direct phage antibody selection on primary CRC may identify antigens with promising tissue distribution for immunotargeting of cancer.

On the basis of their expression pattern, the antigens targeted by the selected antibody fragments differ from each other, but do not correspond to any of the known colon tumour-antigens c-ERB-2 [27], CEA [28] or Ep-CAM [29]. This was confirmed in enzyme-linked immunosorbent assay (ELISA) using the purified proteins (data not shown). Whether the exact epitope recognised by the selected antibodies is composed of a carbohydrate structure has not yet been assessed. Therefore, it cannot be excluded that the selected antibodies are directed to cancer-associated carbohydrates (e.g. TAG-72/Sialyl-Tn [30]). However, this seems very unlikely, since the immunoreactivity of the selected antibodies could be restored by treatment with a reducing agent (data not shown), which does not affect carbohydrate structures. Thus, the epitopes recognised by the selected antibodies are most likely composed of proteins and not of glycolipids or carbohydrate structures of glycoproteins. Further characterisation of these antigens using appropriately treated cell preparations is expected to reveal their molecular nature of these antigens.

In conclusion, we have shown that phage antibody display provides a powerful means to quickly select anti-tumour antibodies that show moderate cross-reactivity with normal tissues and that this procedure may identify suitable targets for immunotargeting of CRC.

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