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ORIGINAL ARTICLE

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Immunoglobulin genes expressed by B-lymphocytes infiltrating cervical carcinomas show evidence of antigen-driven selection

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Abstract Lymphocyte infiltration is often present in cervical cancer lesions, possibly reflecting an ongoing (but ineffective) immune response to the tumour. B-lymphocytes are the predominant lymphocyte infiltrate in pre-malignant cervical lesions, where they are thought to comprise the host immune response to active human papillomavirus (HPV) infection. Although B cells are less frequently detected in cervical tumours, a high proportion of terminally differentiated plasma cells expressing tumour-specific immunoglobulin (Ig) remain. The antigen specificity and functional significance of the antibody response to cervical tumours is unknown. As part of a study to characterise the antibodies expressed by the tumour-infiltrating B cells (TIL-B) in cervical tumours using antibody phage display, we examined expressed Ig gene sequences to determine if there was molecular evidence of a selective response to antigenic changes in the transformed epithelial cells. We found that biased variable region gene usage by the B cells and the rate of somatic hypermutation in the rearranged Ig heavy chain variable regions (VH) both indicated antigenic selection of the B cells. We also found evidence of affinity maturation, as indicated by the detection of antibodies of the IgG1, IgG2 and IgA isotypes, and possible clonal selection of the Ig receptors. These data support the notion that B-lymphocytes and plasma cells infiltrating cervical carcinomas are the result of an antigen-induced response to HPV infection or transformation.

Keywords Cervical carcinoma · HPV · Antibody response

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Introduction

The initiating event in the development of cervical carcinoma is thought to be infection of cervical epithelium with human papillomavirus (HPV) [48]. Although in almost all cases, HPV infection is cleared following activation of the host immune system, the virus can sometimes persist leading to a malignant progression of disease. This appears to depend on several factors, including: (a) the infecting virus type, with the so called "high-risk" virus types HPV-16 and HPV-18 predominantly detected in cervical tumours [34]; (b) exposure to environmental cofactors, such as cigarette smoke [26]; (c) the ability of the virus to avoid the immune system [20]; and (d) the genetic background of the individual, which may influence the host immune response [28].

The specific mechanisms that contribute to the host immune system-mediated clearance of HPV-infected cells, including tumours, are not clear, but both antibody and cell-mediated responses are implicated. Both $CD4^+$ and $CD8^+$ T-lymphocytes are thought to be major effectors against HPV-infected cells, as demonstrated by animal models and patient studies [19]. T-lymphocytes also appear to be important effector cells against HPV-associated tumours. Cervical tumour lesions are infiltrated with both subsets of T-lymphocytes [4, 16, 18] and T cells recognising HPV proteins can be isolated from patients with high-grade pre-malignant disease and cervical tumours [6, 18, 23, 37]. In addition, as observed in non-malignant disease, infiltration of T

cells in high-grade pre-malignant disease and tumour lesions has been correlated with a more favourable outcome [5, 10, 41, 44]. However, as the development of a cervical tumour presumably means a failure of an effective immune response to clear the virus, the T cells may be ineffective at clearing the infected/malignant cells due to immune escape mechanisms employed by the virus [20] or the tumour cells [11].

The role of antibody in protection against HPV-associated disease is less clear. Both systemic and local (mucosal) antibody can be detected against HPV proteins in patients with cervical disease [15], and antibody induced against HPV capsid proteins by prophylactic vaccination can protect against viral challenge by virus neutralisation [19]. However the extent to which antibody contributes to the resolution of established disease, including HPV-associated tumours is unknown. Antibodies against HPV could conceivably limit the spread and, therefore, extent of disease, and may also be involved in active elimination of virally infected cells [38].

B cells are specifically recruited to sites of HPV-induced pre-malignant (low- and high-grade) cervical lesions, where they are the predominant lymphocyte infiltrate [4, 17, 41, 44] and may reflect a host immune response to active HPV infection [17]. These stages of disease also contain a high frequency of plasma cells and express immunoglobulins (Ig) that bind to the dysplastic epithelial cells [17]. B-lymphocytes are also found in lesions that have progressed to carcinoma, although normally at lower frequency than in the pre-malignant lesions [17]. Plasma cells are also detected in significant numbers in tumours and, as in the pre-malignant lesions, express antibody that can be found bound to tumour cells [17, 31]. It is not known if the tumour-infiltrating B cells (TIL-B) have infiltrated the developing tumour site in response to antigen [either against the persistent HPV virus or against newly expressed tumour-associated antigens (TAA)] or are a component of a normal inflammatory response to the developing tumour: their functional significance is unknown, nor have the antigens to which they bind been identified. However, regardless of their role in protection, antibodies expressed by TIL-B at the tumour site at any stage are a potential source of naturally induced antibody reagents against cervical TAA (including HPV antigens). As part of a study to isolate in vivo induced monoclonal antibodies (mAbs) against cervical TAA using antibody phage display, we have initially characterised the molecular structure of the Ig gene sequences expressed by TIL-B in cervical carcinoma. The results provide the first insight into the nature of the B cell immune response to persistent HPV infection and cervical TAA.

Materials and methods

Tumour samples

Patients undergoing surgery for removal of cervical tumours were included in this study following informed consent. Ethical approval for the study was granted by the Stobhill NHS Trust Ethical Committee. Samples of normal (non-diseased) and tumour cervical tissue, and when available, non-diseased lymph node (from the same patient) were either snap-frozen in liquid nitrogen or fixed in 10% formaldehyde immediately following surgical removal. Confirmation of cervical epithelial malignancy was obtained by histological analysis. All of the patients included in this study were diagnosed with Stage 1B squamous cell carcinoma of the cervix [40]. The patients had not undergone any previous treatment before surgery, and had no clinical evidence of any ongoing concurrent disease.

Detection of infiltrating lymphocytes by immunohistology

Consecutive sections (5 μm) of fixed tumour tissue were cut and prepared for staining using the automated procedure of the Department of Pathology, Royal Infirmary, Glasgow. Sections were differentially stained with haematoxylin and eosin, or immunostained with anti-CD20 (pan B cells) and anti-CD3 (pan T cells) monoclonal antibodies (DAKO, Cambridgeshire, UK). Bound antibody was detected using the EnVision-HRP secondary antibody and substrate kit (DAKO). Sections were counterstained in haematoxylin.

Extraction of cervical epithelial RNA and genomic DNA

For RNA extraction, 100 mg of tissue was ground in liquid nitrogen using a mortar and pestle, transferred into RNAzol (Biogenesis, UK) and total RNA extracted according to the manufacturer's instructions. RNA pellets were dissolved in RNase-free water, then treated with DNA-free (Ambion, AMS Biotechnology, UK) to remove any contaminating DNA, and stored in aliquots at –70 °C. To extract genomic DNA, 25–50 mg of tissue was extracted using a QiaAmp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at –20 °C. All tissue manipulations were carried out under Class II safety containment, and all non-disposable equipment was washed and treated with DNAZap and RNAseZap solutions (Ambion, UK) between samples.

PCR detection of HPV DNA

HPV E6/E7 transcripts were amplified from cervical tumour DNA (1–3 μg) using 1 U of HotStar *Taq* polymerase (Qiagen, Germany) and 200 mM dNTPs in 50 μl. Primers for amplification to detect low risk HPV DNA (HPV6/11) were as follows: E6A (200 pM), 5′-ACCGAAAACGGTCGAACCGA-3′; E7C (100 pM), 5′-GGAG GTTGCAGGTCTAGTAC-3′ (550 bp product). To detect high risk HPV DNA (HPV16/18): E6A (200 pM); E6B (100 pM), 5′-AATAATGTCTTTATTCACTA-3′ (307 bp) [47]. Primers to detect β-globin were included as a positive control: BGF, 5′-GGTTGGCCAATCTACTCCCAGG-3′; BGR, 5′-TGGTCTCC TTAAACCTGTCTTG-3′ (685 bp product). Following 15 min at 95 °C to activate the *Taq* polymerase, PCR conditions were 40 cycles at 94 °C for 1 min, 55 °C (40 °C for reactions containing the E6B primer) for 1.5 min, and 72 °C for 1 min; followed by a 10 min incubation at 72 °C. The resulting products were electrophoresed on a 1.5% agarose gel.

RT-PCR amplification of Ig variable region sequences expressed by TIL-B

Total RNA (10–30 μg), 16 μM oligo dT primer (Promega, USA), and diethyl pyrocarbonate-treated water were aliquoted into a DNAse-free 0.2 ml tube, and heated to 70 °C for 10 min, then chilled on ice. To the reaction were added (final concentrations): 1×reverse transcriptase (RT) buffer and 10 mM DTT (Life Technologies, Scotland), 2 mM dNTPs, 200 U RNasin (Promega, USA), and 500 U of MMLV-RT (Life Technologies). Following a 10 min incubation at 22 °C, the tube was incubated for 1 h at

37 °C. Oligonucleotides for PCR amplification of the entire human Ig VH and VL gene families [3] were synthesised and purified by RPLC before use (Interactiva, Germany). This set of primers has been designed for the construction of human Fab libraries in pComb3 expression plasmids [2]. PCR amplification of Ig cDNA was as follows (20 µl): 1×HotStar *Taq* polymerase buffer, 0.2 mM dNTPs, 1 U Hot Star *Taq* polymerase, 10 pM of each oligonucleotide primer and 1 µl of the cDNA reaction. Controls for contamination included tubes with no cDNA, detection of genomic DNA contamination (equivalent amount of non-reverse-transcribed RNA), and amplification of actin cDNA. Following 15 min at 95 °C, the PCR reactions were: 35 cycles at 95 °C for 30 s, 52 °C for 50 s, and 72 °C for 1.5 min; followed by a 10 min incubation at 72 °C. Ten microlitres of the PCR products were electrophoresed on 1.5% agarose gels.

Cloning and sequencing of heavy chain variable region sequences

Heavy chain variable region (VH) PCR products were purified using a Qiaquick Gel Extraction kit, then digested with *Spe*I and *Xho*I and ligated into pComb3H-his, a derivative of pComb3 [2], to which a six-histidine affinity tag has been added in frame to the VH insertion site (P O'Brien, unpublished data) and transformed into *E. coli* XL1 Blue. Plasmid DNA was prepared from a random selection of the resulting VH clones for each patient and the nucleotide sequence of both strands of the VH inserts was determined using the following oligonucleotide primers: pelBF, 5′-CTACGG CAGCCGCTGGATTG-3′; gIII, 5'-TCGGCATTTTCGGTCA-TAGC-3′. Sequencing reactions were carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, UK) and an ABI 377 DNA Sequencer (University of Dundee Sequencing Service, Scotland). The sequences were analysed and assigned to their closest germline derivations using V-BASE (http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO); or IMGT, the International ImMunoGeneTics database (http://imgt.cnusc.fr:8104); initiator and co-ordinator: Marie-Paule Lefranc, Montpellier, France. The sequences were aligned and sequence homologies calculated from the first nucleotide following the oligonucleotide primer sequence in the framework (FR)1 region to the second last nucleotide of the FR3 region. Mutations at the last nucleotide position of the sequenced fragment were excluded from the analysis, as they might result from nucleotide deletion at the joining sites. The frequency of replacement mutations (R) in the complementarity determining regions (CDR) and FR regions for each of the antibodies was calculated with respect to its closest germline gene [9]. The probability that R mutations were occurring at a frequency above or below the expected random frequency was calculated in a binomial distribution model using the expected number of R mutations in the germline gene, the actual number of observed R mutations in the Fab sequences, and the probability of R mutations localising to the CDR or FRs. Silent (S) mutations occurring within a R codon were excluded when calculating R and S ratios. A p value of less than 0.05 indicated that the R mutations had occurred in a non-random fashion.

Results

We randomly selected for inclusion in this study four patients who had histologically confirmed squamous cell carcinoma of the cervix. We firstly confirmed that the tumours were infiltrated with lymphocytes, as previously shown [4, 16, 17]. The results from one patient (742) are shown in Fig. 1. Differential staining with haematoxylin and eosin detected the presence of an intense lymphoid infiltrate, including plasma cells, into the area surrounding the tumour mass (Fig. 1A). Immunostaining with anti-CD3 antibody showed the presence of T cells both infiltrating into the tumour mass and in the underlying stroma, as expected [4, 16] (Fig. 1B). A dense infiltration of B cells into the tumour mass was also detected, as indicated by CD20 antibody binding. The B cells were mainly restricted to the tumour margins and had not infiltrated significantly into the tumour mass (Fig. 1C). Although variable between patients, this pattern of lymphocyte reactivity is similar to what we (D. Millan, unpublished data) and others [4, 16, 17] have observed in patients with low-grade cervical tumours.

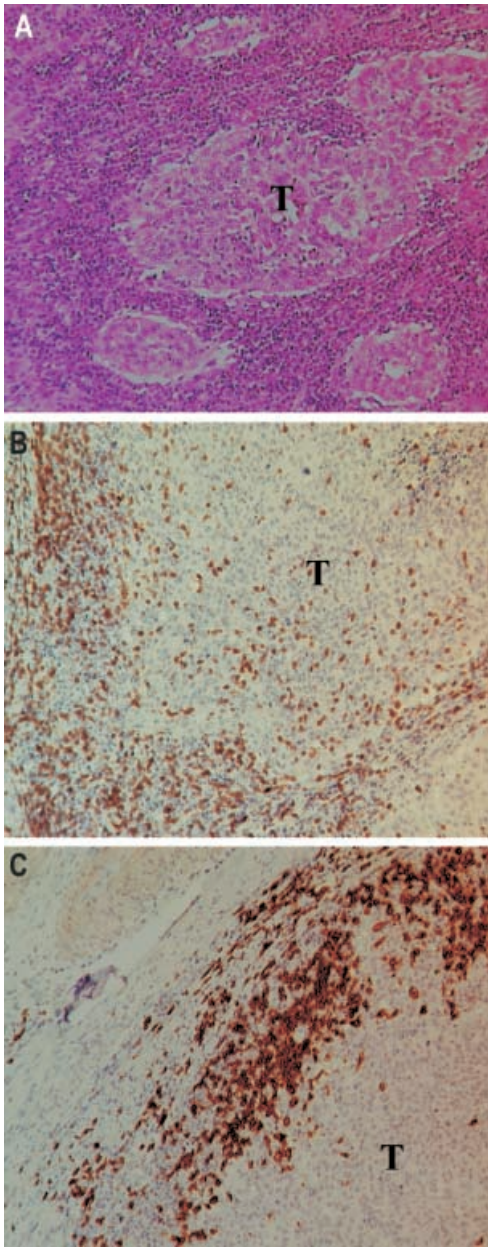


Fig. 1A–C Lymphocyte infiltration in cervical tumours as detected by differential staining and immunohistochemistry. **A** Haematoxylin and eosin (magnification 100×). **B** Anti-CD3 (pan T cell) (200×). **C** Anti-CD20 (pan B cell) (200×). *T* tumour tissue

As the development of malignancy is thought to be associated with persistence of HPV (and therefore may be stimulating an antigenic response), we looked for evidence of HPV infection in the cervical tumours by detection of viral sequences. As shown in Fig. 2, we were able to detect HPV DNA sequences by PCR amplification with primers that detect the "high risk" virus types commonly associated with cervical tumours (HPV-16 or HPV-18), but not low risk HPV DNA (associated with non-malignant HPV infections) in two of the four patients tested. In one of the patients (742), HPV DNA was amplified from the tumour, but not in normal non-diseased cervical epithelium from the same patient. Non-diseased tissue was not available for patient 690. Although not a quantitative PCR reaction, the intensity of the amplified bands suggest a relatively low copy number of the infecting HPV genome, similar to that found in the cervical tumour cell line HeLa, rather than a high genome copy number as detected in CaSki cells. Repeated PCR analysis of the DNA samples yielded identical results.

During B cell development, germline-encoded gene segments are selected and rearranged to form functional Ig sequences that encode the variable and constant regions of the Ig heavy and light chains that make up an antibody molecule. The variable regions, which together form the antigen-binding fragment (Fab) of the Ig, are formed by the splicing of a variable (V) gene and a joining gene (J), and in the case of heavy chains, a diversity gene (D). The human V genes have been cloned and sequenced, and assigned into families on the basis of their nucleotide similarity [12]. In order to determine the range of light chain (lambda and kappa) and heavy chain variable region V gene families being expressed by the TIL-B, we extracted total RNA from the cervical tumours and non-diseased tissue and subjected it to RT-PCR amplification of the Ig variable regions. In all four patients, a more diverse range of Ig variable region sequences were being transcribed in the tumour tissue compared to the normal (non-diseased) epithelial tissue, which presumably reflects a specific infiltration of B cells into the tumour tissue. Figure 3 shows the results from two patients, 946 and 742. Similar results were obtained for the remaining two patients, although patient 555 had a very low level of Ig expression in the normal tissue (data not shown). Repeated PCR amplifications using the same tumour RNA gave identical results. Amplification of the heavy chain variable region segments (VH), which incorporate the CH1 (Fd) portion of the constant region of the heavy chain, showed that different isotype transcripts, including IgG1, IgG2 and IgA, were expressed (Fig. 3).

We chose one patient, 742, who was positive for high risk HPV DNA to further characterise the Ig sequences expressed by the TIL-B. As part of the procedure to construct a phage-antibody library, the

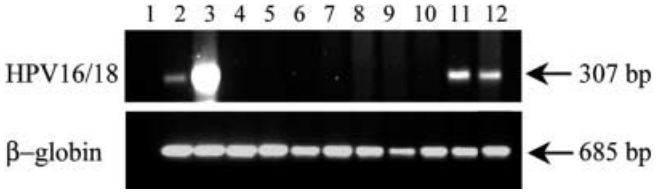
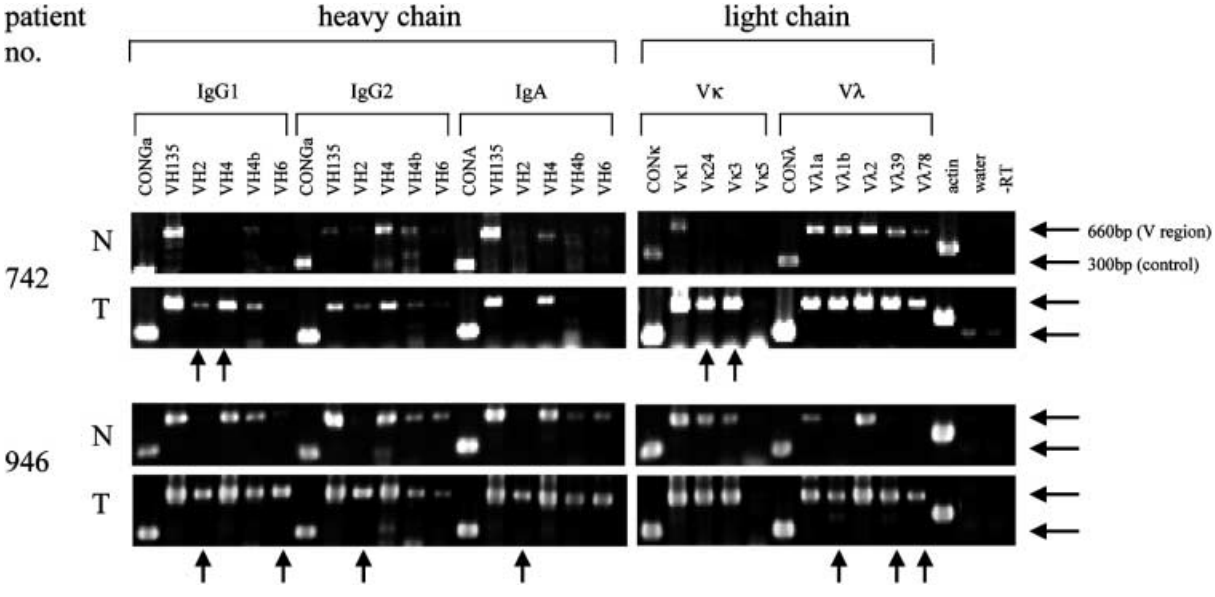


Fig. 2 PCR amplification of HPV DNA sequences from genomic DNA isolated from cervical tumour cell lines and patient cervical epithelium. *Lane 1* no DNA, *lane 2* HeLa (HPV18), *lane 3* CaSki (HPV16), *lane 4* C33 A (HPV negative), *lane 5* HT29 (HPV negative), *lane 6* patient 946 undiseased tissue (N), *lane 7* 946 tumour tissue (T), *lane 8* patient 555 N, *lane 9* 555 T, *lane 10* patient 742 N, *lane 11* 742 T, *lane 12* patient 690 T. Non-diseased tissue was not available for patient 690

Fig. 3 RT-PCR amplification of variable region gene families and Ig isotype expressed by TIL-B in undiseased (normal) and tumour cervical epithelium. The results from two patients (742 and 946) are shown. *Arrows* indicate expression of V gene families only found in tumour tissue



VH sequences were cloned into pComb3H-his. The nucleotide sequence of 27 randomly selected resulting clones was determined and the V, D, and J gene usage assigned to their closest germline derivative using V-BASE or IMGT software (Table 1). The VH region was chosen for this analysis, as it is this chain, in particular the CDR3 region, that mostly determines the antigen specificity of an antibody [45]. All 27 clones encoded functional heavy chain variable regions with in-frame rearrangements of the CDR3 region, although one clone did not appear to encode a D segment. D regions were not assigned when a confident alignment to the germline sequence could not be made due to extensive mutation.

A comparison of VH family usage revealed that the VH3 gene family was used at the highest frequency by the TIL-B (44.4%), which correlates with the dominance of VH3 family usage in induced antibody responses in vivo [35] and in adult peripheral blood [21] (Table 2). However, both the VH4 (37.0%) and VH6 (11.1%) gene families appeared to be over-represented in our analysis, and, although the VH1 gene family is normally detected frequently in antibody responses in vivo, we did not detect any VH1 usage by the TIL-B. When the V gene usage was examined at the level of gene loci, we also found that particular loci were predominantly used (Table 1). In particular, V genes 4–34, 4–59 and 3–23 were represented at a high frequency, all of which have been shown to be preferentially used in human antibody responses [35]. In addition, gene loci 3–07, 3–30 and 6–01 were also used repeatedly by the TIL-B. J region usage appeared to be random, which has been previously observed and it is not thought to be a basis for antigen selection [35].

The sequence analysis also allowed us to determine the isotype of the Ig genes expressed in the tumours at a molecular level. The predominant isotype detected was IgG1 (51.9%), followed by IgG2 (29.6%) and IgA

Table 1 Assignment of closest germline variable region gene loci and antibody isotype of 27 heavy chain sequences isolated from patient 742 (high-risk HPV+). Germline genes were assigned using V-BASE or IMGT alignment software (see Materials and methods). *NA* not assigned due to uncertainty in sequence (score less than 50), – no sequence identified

Clone	family/locus	D region	J region	isotype
01	VH2–05	NA	J4–02	IgG1
06	VH2–05	NA	J4–02	IgG1
07	VH3–07	NA	J3–02	IgA1
23	VH3–07	–	NA	IgG2
24	VH3–07	D6–25	J4–02	IgG1
05	VH3–15	NA	J6–02	IgG1
17	VH3–21	D1–07	J3–02	IgG2
22	VH3–21	D1–07	J3–02	IgG2
04	VH3–23	D1–26	J4–02	IgA2
16	VH3–23	NA	J4–02	IgG2
4.4	VH3–23	NA	J4–02	IgG2
4.1	VH3–30	D3–22	J4–02	IgG1
4.2	VH3–30	NA	J3–02	IgA2
14	VH3–30	NA	J3–02	IgA2
4.5	VH4–34	D3–10	J6–02	IgA2
08	VH4–34	NA	J6–02	IgG1
18	VH4–34	D2–02	J4–02	IgG1
19	VH4–34 (DP71)	NA	J4–02	IgG1
20	VH4–34	NA	J3–01	IgG2
11	VH4–39/4–61	NA	J4–02	IgG2
02	VH4–59	D4–11/D4–04	J2–01	IgG2
4.3	VH4–59	D6–13	J1–01	IgG1
13	VH4–59 (DP71)	NA	J6–02	IgG1
21	VH4–59	D2–02	J4–02	IgG1
09	VH6–01	D3–10	J3–02	IgG1
12	VH6–01	D2–15/2–08	J6–02	IgG1
15	VH6–01	D2–02	J6–02	IgG1

Table 2 Heavy chain variable region families used by TIL-B clones ($n = 27$) isolated from a cervical tumour lesion and comparison to VH gene family usage in immune responses in vivo against protein antigens (adapted from Ohlin and Borrebaeck [35])

V gene family	% representation in repertoire			
	TIL-B	Hybridomas	Phage Ab	Adult PB
VH1	0	26	35	11
VH2	7.4	4	0	3
VH3	44.4	40	47	52
VH4	37.0	22	13	19
VH5	0	8	2	10
VH6	11.1	0	3	5

(18.5%). This distribution correlates with antibody isotypes detected in non-diseased cervix and reflects the mucosal nature of the tissue [14].

Following exposure to antigen, Ig gene sequences are modified by base changes in the nucleotide sequence in order to produce a "better fit" or higher affinity antibody. This process, known as somatic hypermutation, together with proliferation of the resulting B cell clones is called affinity maturation, and is an ongoing process in response to antigenic stimulation. The extent of somatic hypermutation was determined in the 27 VH clones by assessing the number of nucleotide changes over the variable region sequence (FR1 to FR3) compared to their assigned germline derivatives. All of the clones showed a degree of deviation from the germline sequence, with nucleotide homologies ranging from 86–98.6% (Table 3). This mutation rate is similar to that observed for other antibodies and is highly suggestive of antigenic selection, compared to the rate of mutation found in non-selected (IgM) antibodies (> 99% homologous to germline) [35].

The distribution and frequency of nucleotide mutations within a V gene can be used to differentiate between those antibodies with random mutations and those driven by antigen selection of a B cell clone. Thus, the silent (S) and replacement (R) mutations (i.e. nucleotide replacements that result in amino acid changes) occurring in the CDR or FR of each V gene were used to calculate the probability that the R or S mutations had occurred in a non-random manner (Table 3). The majority of the V genes analysed (20/27) were shown to have reduced R mutations within the FR region, which is indicative of an antigen driven B cell selection. Two of these antibodies also had elevated R mutations within their CDR region, which confirms the antigen driven nature of the response.

We also compared the deduced amino acid sequences over the CDR3 region of each of the 27 clones. This region is made up of the 3' end of the variable region, spliced to the D and J segments, and is the region that mostly determines antibody specificity [45]. Identical or closely related amino acid sequences in this region are suggestive of similar binding specificities, and along with the nucleotide sequence, can indicate clonality in the induced antibody response. We identified 3 pairs of clones (01 and 06; 17 and 22; 4.2 and 14) that differed in only one amino acid in this region, all caused by a single nucleotide change (Table 4). In addition, a further pair of clones (4.4 and 16) shared identical CDR3 regions at the amino acid and nucleotide level. The nucleotide sequence of all of these pairs of clones was also very similar over the entire FR1-FR3 region, with only 1 or 2 nucleotide differences occurring in the FR or CDR1 regions (data not shown).

Table 3 Analysis of the heavy chain variable region somatic mutation rate. The number of replacement (*R*) and silent (*S*) mutations in the FR and CDR of each antibody were determined and compared to the assigned germline sequences. A *p* value of less than 0.05 indicates that the R mutations have occurred in a non-random fashion

Clone	Total No. mutations	% Homology	CDR R mutations (expected)	FR R mutations (expected)	CDR R:S ratio	FR R:S ratio	*P* (CDR)	*P* (FR)
01	14/277	94.9	3 (2.55)	5 (8.07)	3:5	5:2	0.24	0.06
06	20/277	92.8	5 (3.64)	8 (11.52)	5:2	8:2	0.15	0.05
07	10/272	96.3	3 (1.87)	2 (5.74)	3:0	2:4	0.18	0.02
23	19/272	93.0	5 (3.55)	5 (10.91)	5:2	5:3	0.15	0.004
24	10/272	96.3	1 (1.87)	4 (5.74)	1:1	4:5	0.29	0.14
05	37/278	86.7	6 (7.05)	12 (20.69)	6:3	12:13	0.16	0.002
17	13/272	95.2	2 (2.36)	2 (7.43)	2:2	2:7	0.28	0.002
22	14/272	94.9	2 (2.55)	4 (8.01)	2:1	4:6	0.27	0.02
04	19/272	93.0	6 (3.32)	5 (10.91)	6:0	5:12	0.06	0.005
16	37/272	86.4	6 (6.48)	10 (21.24)	6:5	10:12	0.17	0.0002
4.4	38/272	86.0	6 (6.65)	10 (21.81)	6:5	10:9	0.17	7.7×10^{-5}
4.1	9/272	97.0	2 (1.60)	3 (5.17)	2:1	3:2	0.29	0.10
4.2	25/272	90.8	6 (4.43)	11 (14.35)	6:3	11:2	0.13	0.06
14	25/272	90.8	6 (4.43)	12 (14.35)	6:3	12:2	0.13	0.10
08	23/272	91.5	3 (4.23)	5 (13.14)	3:2	5:9	0.19	0.0005
18	34/272	87.5	6 (6.26)	5 (19.43)	6:3	5:15	0.18	3.6×10^{-7}
19	4/272	98.5	0 (0.74)	0 (2.29)	0:0	0:2	0.44	0.03
4.5	11/272	96.0	2 (2.02)	4 (6.29)	2:1	4:0	0.30	0.09
20	7/272	97.4	1 (1.29)	1 (4.00)	1:1	1:1	0.38	0.02
02	11/269	95.9	3 (1.95)	3 (6.21)	3:1	3:2	0.19	0.04
13	18/269	93.3	4 (3.20)	4 (10.15)	4:0	4:8	0.20	0.003
21	31/269	88.5	9 (5.50)	12 (17.49)	9:2	12:6	0.05	0.02
4.3	21/269	92.2	6 (3.73)	7 (11.85)	6:0	7:6	0.09	0.019
11	42/275	84.7	8 (7.90)	11 (23.22)	8:3	11:9	0.16	9.3×10^{-5}
09	17/281	94.0	6 (3.40)	4 (9.34)	6:1	4:4	0.07	0.007
12	22/281	92.5	8 (4.41)	5 (12.09)	8:3	8:8	0.04	0.002
15	4/281	98.6	0 (0.80)	2 (2.20)	0:0	2:2	0.41	0.37

As the success of this type of analysis depends on the fidelity of the PCR amplification reaction, we took a number of steps to reduce the chance of *Taq* polymerase error during PCR amplification of the transcribed sequences. Our amplification protocol involved a single PCR reaction under optimal amplification conditions [42, 49], and we performed independent amplifications from the same RNA samples that resulted in Ig clones that encoded almost identical nucleotide sequences, which suggests an accurate amplification process [49]. In addition, following sequencing of the heavy chain constant regions of the 27 clones (a total of over 9000 bases), we detected only two potential errors, as indicated by unique sequence substitutions, which agrees with the estimated error rate of *Taq* polymerase [42]. We are, therefore, confident that these sequences represent accurate copies of the transcribed Ig genes.

Discussion

Lymphocyte infiltration is well documented in tumours of various histological types, and both experimental and clinical data suggest that lymphocyte presence in solid tumours may be a sign of host resistance. The nature of the lymphocyte infiltrate has been studied and in most tumours predominantly consists of T-lymphocytes, with fewer B-lymphocytes, macrophages and natural killer cells. As T cells are thought to be major immune effectors against tumour cells, studies determining the nature of the immune response to tumours have focused on defining the T cell antigens and the effector cells that recognise them [7].

In contrast, very little is known about the nature and functional significance of antibody responses to a tumour [29]. Serum antibodies from tumour patients can recognise autologous tumour cells, and can be used to define the antigens expressed on human tumour cells [43]. In addition, B cell and plasma cell infiltrates are found in tumours of various histological types and their presence has been associated with a more favourable outcome in some tumours. It has also been shown that TIL-B can express antibodies (IgG) that recognise autologous and allogeneic tumour targets of the same histological type [25, 36, 46]. Therefore, there is an increasing body of evidence that antibody expressed by TIL-B and their corresponding plasma cells is common in tumours, although the antigens to which they are directed have largely not been defined. This is also the case for cervical carcinoma, where the infiltration of B cells and plasma cells into both pre-malignant and malignant cervical lesions and the concurrent expression of anti-tumour antibody suggest an active response to antigenic stimulation. Analysis of Ig gene expression at the molecular level can provide detailed information on the nature and clonality of the host antibody response [33,35]. In this study, we used RT-PCR to amplify the Ig genes expressed by TIL-B in cervical tumours to assess at a molecular level the evidence for these cells being a component of a specific immune reaction to the tumour.

The selection of V genes by B cells is not random, and limited use of the Ig V gene repertoire in response to specific antigen has been demonstrated [35]. For example, a marked reduction in use of the VH3 family has been observed in the antibody response against HIV [32]. Although VH3 gene family usage appeared to correlate with the frequency of use in antigen-induced antibody responses, we did detect other changes in V gene usage by the TIL-B in cervical carcinoma. The most evident changes were an absence of VH1 gene expression, which is normally used at a relatively high frequency, and an increased usage of the VH6 gene family. Analysis of V gene loci usage by lymphocyte receptors can also indicate the dominance or clonal nature of an immune response to a particular antigen, and its use has been demonstrated in T cell receptor gene selection in response to pathogens [30] and tumours [22, 39]. Apart from B cell lymphomas [1], the analysis of Ig receptor V gene usage in B cells responding to both pathogens and tumours [27] is an emerging field. However, the dominance of specific VH gene loci induced by antigens has been reported, including tetanus toxoid (3–23/DP-47) and the AD-1 protein of cytomegalovirus (5–51) [35]. Although we analysed a small population of VH genes expressed by TIL-B in cervical tumours, we also found the predominant usage of V gene loci, in particular 4–34 (used by 18% of the isolated antibody clones) and 4–59 (almost 15%). Therefore, biased V gene usage by the TIL-B may indicate a specific response to dominant antigen(s) in the tumour.

Further evidence that the Ig sequences originated from B cells undergoing antigen driven affinity maturation was demonstrated by sequence analysis of the TIL-B clones. Firstly, determination of the extent of nucleotide sequence deviation from the germline V gene

Table 4 Comparison of the amino acid (aa) sequence of the CDR3 (VDJ) regions in similar TIL-B clones. Amino acid differences are in *bold type* and *underlined*

clone	VH loci	VDJ aa sequence	Isotype
742–01	2–05	CAR LRIVQGASDY WGQG	IgG1
742–06	2–05	CAR LRIVQGAGDY WGQG	IgG1
742–17	3–21	CAR GYTGNYRAAFDI WGQG	IgG2
742–22	3–21	CAG GYTGNYRAAFDI WGQG	IgG2
742–16	3–23	CAR SPRVSIFSLYFDS WGQG	IgG2
742–4.4	3–23	CAR SPRVSIFSLYFDS WGQG	IgG2
742–4.2	3–30	CAR KDSYYALDI WGQG	IgA
742–14	3–30	CAR EDSYYALDI WGQG	IgA

sequences showed that all of the clones expressed highly mutated gene sequences consistent with antigenic selection. When we analysed the significance of amino acid replacement (R) mutations in the FR and CDR regions of the V genes, we found that negative selection had occurred in the FR regions, which is typical feature of affinity matured antibodies [9]. Interestingly, although the CDR or hypervariable regions are normally subject to a higher rate of R mutations during antigen selection, as these regions encode the amino acids that form the antigen binding groove, only a small proportion of the TIL-B sequences had significantly elevated R mutations within the CDR regions. This has also been observed previously in human Ig sequences specific for the tumour antigen p53 [13] and the *Haemophilus influenzae* type b capsular polysaccharide [24]. This disparity is most likely to reflect the emergence of optimal CDR amino acid sequences over time following extended stimulation of B cells by TAA, resulting in less selective pressure for R mutations to occur over S mutations, leading to reduced statistical significance [24]. In contrast, R mutations within the FR would be continually suppressed to maintain structural integrity of the antibody and over time would become increasingly significant, as detected in our study and others [13, 24]. Secondly, although we presumably sampled only a small proportion of the antibody clones infiltrating the tumour, the nucleotide sequence analysis revealed that we had isolated antibody clones with very similar or identical CDR3 regions, which is suggestive of binding to the same antigen [45]. Although these pairs of clones were not identical at the nucleotide level, the very high degree of overall homology suggests a degree of clonal selection in the tumour environment. The minor sequence differences may reflect the continued accumulation of nucleotide mutations in the CDR3 due to the ongoing process of affinity maturation.

Our results are suggestive of an induced antibody response to tumour antigens expressed in cervical carcinoma. It is not known if the TIL-B cells infiltrating tumours are residual B cells remaining from an early response to active HPV infection and therefore express antibody that recognises HPV proteins; or if they have moved into the tumour environment in response to newly formed or expressed TAA due to malignant cellular changes. The nature of the antigenic targets has not been determined, but could include HPV proteins, such as the major transforming proteins E6 and E7, that do not appear to be exposed to the immune system until the development of malignancy [8]. We were able to detect HPV DNA sequences in two out of the four patients in this study; however, it is likely that HPV is also present in the remaining two patients, as other studies have demonstrated the presence of HPV in over 99% of cervical tumours [34]. It is possible that the sensitivity of our PCR system was not sufficient to detect very low copy numbers of HPV, that the tumour did not contain HPV at that particular sample site, or that the oligonucleotide primers were unable to amplify the infecting HPV type in those patients. However, at this stage of our study the nature of the antigenic targets is speculative.

Several studies have shown by immunohistology that TIL-B in cervical carcinoma express immunoglobulins, including IgG and IgA [17, 31]. Our study confirms this observation, and extends it to the molecular level. We found that the predominant Ig isotype expressed by the TIL-B was IgG1 (54%). The expression of this isotype is associated with the development of an antibody response aided by T cells of the type 2 (Th2) phenotype. However, an effective anti-viral response requires the induction of a type 1 (Th1) T cells, which in addition to stimulating anti-viral effector T cells, provide help for the induction of antibodies of the IgG2 isotype. We found that 29% of the isolated Igs were of the IgG2 isotype, which may therefore be indicative of at least a partial anti-viral immune response. We also observed antibodies of the IgA isotype, both IgA1 and IgA2 ($\sim$17%), which corresponds with the mucosal location of the TIL-B. Although IgA antibodies are the predominant antibody isotype found at mucosal surfaces, where they play a major role in the neutralisation of pathogens, their role in protection against viral infection, in particular HPV, is unclear. However, the identification of these isotypes indicates Ig class switching from IgM, which is indicative of a memory response to antigen. The functional significance of the antibody response is yet to be determined, but the binding of antibody to tumour cell targets could stimulate cytotoxic mechanisms, such as activation of the complement system or antibody-dependent cellular cytotoxicity (ADCC), which has been shown to be involved in tumour clearance including HPV-associated tumours [38].

We have constructed several recombinant phage-antibody libraries using Ig heavy and light chain variable region sequences amplified from TIL-B infiltrating cervical carcinomas, and we are currently screening these libraries against cervical tumour cells in an attempt to isolate tumour-specific human mAbs. In addition to their potential as immunodiagnostic markers and as the basis for immunotherapy strategies, characterisation of these antibodies, including the identification of the antigens to which they bind, will provide further information on the nature and extent of the B cell response to cervical tumours.

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