Monoclonal antibody to cytokeratin for use in routine histopathology

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SUMMARY CAM 5.2 is a murine monoclonal antibody, raised against the colon carcinoma cell line HT29, which recognises lower molecular weight intracellular cytokeratin proteins within secretory epithelia. Extensive indirect immunohistochemical studies have confirmed that this antibody stains formalin fixed (and freshly frozen) normal and malignant human tissue in a consistent manner. Reliable staining of conventionally processed pathological tissues provides more accurate identification and staging of human malignant epithelial diseases.

The aim of this study was to look for markers of colonic carcinoma. The most interesting monoclonal antibody raised, however, proved to be a reliable marker of epithelial tissues. Antisera and monoclonal antibodies directed against the family of cytokeratin polypeptides ranging from 70 000 to 40 000 daltons have a recognised place in surgical pathology.¹⁻⁴ Most workers, however, have used freshly frozen tissue specimens or tissues fixed by unconventional methods due to alteration or loss of antigenic determinants by routine tissue processing.

Antibody CAM 5.2 is an IgG2a murine monoclonal immunoglobulin generated from a fusion of a mouse myeloma cell line with lymphocytes from a mouse immunised with colorectal carcinoma cells. The antibody, as characterised by immunoblotting, immunoprecipitation, and immunofluorescence, identifies the lower molecular weight cytokeratin proteins (50 000, 43 000, and 39 000 daltons). Extensive assessment by immunoperoxidase on formalin fixed paraffin embedded human tissue sections shows reproducibly consistent homogeneous staining. The advantages of CAM 5.2 as opposed to other epithelial markers—for example, EMA and HMFG1 and 2—in surgical pathology and the application of this novel reagent to the immunohistochemical analysis of the “difficult” histopathological tumour are discussed.

Material and methods

CELLS, TISSUE CULTURE, AND TISSUES

HT29, a human colorectal carcinoma cell line

obtained from J Fogh, Sloan-Kettering Institute for Cancer Research, New York, was maintained in medium RPMI 1640 containing 10% fetal calf serum at 37°C in 5% CO₂ in air at 100% humidity.

SW837, SW620, and SW48, are human colorectal carcinoma cell lines originating from different tumours.⁵ They were maintained in Dulbecco's modification of Eagle's medium containing 10% FCS at 37°C in 10% CO₂ in air at 100% humidity.

LS147T, also a human colorectal carcinoma cell line,⁶ was maintained in Dulbecco's modification of Eagle's medium containing 10% FCS.

Bu, a human fibroblast cell line, was maintained in RPMI 1640 containing 10% FCS. Bu was a gift from Dr S Povey, Galton Laboratory, London.

P3/NS1/1-Ag4-1 (NS1), an azaguanine resistant BALB/c myeloma cell line,⁷ was maintained in RPMI 1640 containing 10% FCS and 6-thioguanine (2×10^{-5} M).

Hybridoma CAM 5.2 was initially cultured in RPMI 1640 supplemented with 20% FCS, 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, and 10^{-5} M methotrexate (HAT). Later the hybridoma was weaned off HAT and maintained in RPMI 1640 containing 10% FCS.

Fresh human colonic carcinomas and samples of morphologically normal large bowel distant from the primary tumour were provided by Dr BC Morson, St Mark's Hospital, London. Fresh fetal tissues were supplied by Dr Ruth Nash, New Cross Hospital, London. Fixed tissue sections were supplied by Professor PG Isaacson, Department of Histopathology, University College Hospital Medical School, London, and fixed in unbuffered, 10% formol-saline

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before conventional processing and embedding in paraffin wax.

IMMUNISATION AND PRODUCTION OF HYBRIDOMAS

BALB/c mice were immunised by an initial intraperitoneal inoculation of 2×10^6 live trypsinised HT29 cells suspended in 0.2 ml RPMI 1640 and 0.2 ml complete Freund's adjuvant. Two months and five months later the animals were boosted with 10^6 HT29 cells in 0.2 ml RPMI 1640 intravenously.

Four days after the last injection the spleen was removed aseptically; a single cell suspension was made and fused with 10^6 NS1 myeloma cells using 50% polyethyleneglycol (4000 Merck) in RPMI 1640.⁷ The cells were plated out in six 96 well plates (Linbro, Flow Laboratories Ltd) with mouse spleen cells as a feeder layer overlaid with RPMI 1640 containing HAT and 20% FCS; the cells were cultured at 37°C in 5% CO₂ in air at 100% humidity for 14 days. Screening to identify interesting hybridomas was performed before cloning. Cloning was done by picking single cells and culturing them in individual wells of 96 well plates using mouse spleen cells as a feeder layer, in RPMI 1640, HAT, and 20% FCS at 37°C in 5% CO₂ in air at 100% humidity.

SELECTION OF ANTIBODY PRODUCING HYBRIDS

Assay system

Initial screening was performed by indirect enzyme linked immunosorbent assay (ELISA).⁸ Ninety six well plates (Nunc-Immuno, Denmark) were treated with poly-L-lysine (Sigma) 0.1 mg/ml in phosphate buffered saline (PBS) for 1 h. All reactions were carried out at room temperature, unless otherwise stated. Confluent monolayer cultures of HT29 cells were trypsinised to obtain a single cell suspension, washed three times in PBS, and plated at a density of 10^5 cells in 50 µl PBS per well. To encourage adherence of cells to the base of each well, plates were centrifuged for 5 min at 1500 g and then allowed to stand for a further 10 min. The cells were fixed with 0.025% glutaraldehyde (BDH) in PBS for 15 min before washing twice in PBS. Non-specific binding of monoclonal antibody to the plastic plate was reduced by filling each well with gelatin (200 µg/ml in PBS) for at least 1 h. If not for immediate use plates were stored at this stage at 4°C for up to six weeks. After two washes in PBS, endogenous peroxidase activity in the cells was blocked with 0.1% phenylhydrazine (Sigma) in PBS for 1 h. Subsequent washes between each stage consisted of two washes with PBS and one wash with 0.2% Tween 20 in PBS. Twenty five microlitre aliquots of tissue culture supernatant were applied in duplicate for 2 h. Next, 25 µl goat antimouse immunoglobulin diluted

1/20 in 200 mM Tris, pH 7.6, with 10% normal goat serum was added for 30 min. After this, 25 µl of peroxidase-anti-peroxidase (PAP) complex diluted 1/100 in 200 mM Tris, pH 7.6, was added for 30 min. (Goat antimouse immunoglobulin and PAP complexes were obtained from Central Laboratories van de Bloedtransfusiedienst, Amsterdam). The reaction was visualised using 100 µl volumes of 20 mg o-phenylenediamine (Sigma) dissolved in 10 ml citrate buffer (22.5 g disodium hydrogen orthophosphate and 5.6 g citric acid in 1 litre of distilled water, pH 6.0) containing 5 µl hydrogen peroxide; the reaction was left to develop over 30 min in the dark. Absorbance was measured at 450 nm with a Titertek multiskan photometer.

The second screen was performed on freshly frozen tissue sections by the indirect immunoperoxidase method.⁹ Three millimetre cubes of fresh human colonic carcinomas and samples of morphologically normal large bowel distant from the primary tumour were snap frozen in a beaker of isopentane suspended in a freezing bath of acetone and dry ice and stored at -70°C before use. Frozen sections not less than 6 µm were cut on to microscope slides pre-coated with 0.1% poly-L-lysine and allowed to dry in air for 30 min at room temperature. Sections were fixed in fresh acetone for 30 min before washing in Tris buffered saline (TBS), pH 7.6, (605 mg Tris, 8 g NaCl in 1 litre of distilled water). Sections were incubated with unconcentrated monoclonal antibody supernatant for 30 min at room temperature in a humid chamber. After washing with TBS, sections were incubated for 30 min at room temperature in a humid chamber with peroxidase conjugated rabbit antimouse immunoglobulin (DAKO) diluted 1/50 in TBS. Sections were washed again in TBS and the peroxidase reaction was developed over 10 min using a freshly prepared, filtered solution of diaminobenzidine (Sigma, Isopac) (6 mg in 10 ml of 200 mM Tris, pH 7.6, containing 0.03% hydrogen peroxide). Mayer's haematoxylin was used to counterstain the sections before dehydrating and mounting in the conventional manner.

Assay of supernatants

Two weeks after the fusion, when proliferation could be detected microscopically, the unconcentrated tissue culture supernatants were screened by ELISA. Strongly positive antibodies were selected and screened on matched pairs of freshly frozen tissue sections.

IMMUNODIFFUSION

The immunoglobulin class was determined by Ouchterlony immunodiffusion¹⁰ in 1% agarose in barbitone buffer (12 g sodium barbital and 4.4 g barbi-

Fig. 1 Protein immunoblot with antibody CAM 5.2.

Fig. 2 Immunoprecipitation after biosynthetic labelling with ^{35}S -methionine. Columns 1, 3, 5, 7 with CAM 5.2; 2, 4, 6, 8 with LE 61.

Fig. 3 Examples of reactivity of CAM 5.2 on formalin fixed paraffin embedded tissue sections using the immunoperoxidase technique. (a) colon carcinoma; (b) tubular adenoma of colon; (c) squamous carcinoma of the lung; (d) pleural effusion from breast adenocarcinoma.

tal in 1 litre distilled water, pH 8.2) using class and subclass specific rabbit antisera (Miles Laboratories, Slough).

PROTEIN IMMUNOBLOTTING

A whole cell lysate was made of each of the colorectal carcinoma cell lines HT29, SW620, SW837, and LS174T and the fibroblast cell line Bu in sample buffer consisting of 2% sodium dodecylsulphate (SDS), 10% glycerol, 80 mM tris-HCl, pH 6.8, and 100 mM dithiothreitol. The proteins were separated by electrophoresis in one dimension on a 5–15% (wt/vol) gradient polyacrylamide gel overlaid with a 3% (wt/vol) polyacrylamide stacking gel. The running buffer was composed of 25 mM tris base, 192 mM glycine, and 0.1% SDS. Prestained protein molecular weight markers (BRL) were run simultaneously. Transfer of the proteins on to nitrocellulose paper was performed overnight at 4°C using the Trans-Blot (Bio-rad) transfer apparatus and a transfer buffer comprising 25 mM tris base, 195 mM glycine, and 20% methanol.¹¹

Non-specific binding of CAM 5.2 to blot was blocked with 3% bovine serum albumin in PBS for 1 h followed by washing in PBS. The blot was incubated with CAM 5.2 for 40 min, washed in PBS, and then incubated for 30 min with peroxidase conjugated rabbit antimouse immunoglobulin 1/50 in PBS. After a further wash in PBS the proteins identified by the monoclonal antibody were visualised using a saturated solution of 4-chloro-1-naphthol (Koch-Light Laboratories Ltd) in ethanol, which was diluted 1/100 in PBS and filtered and contained 0.1% hydrogen peroxide.

IMMUNOPRECIPITATION

Bu fibroblasts (2×10^7) and SW837 cells (2×10^7) were surface labelled with ^{125}I by lactoperoxidase for 10 min. Labelled cells were lysed in lysis buffer (1% Nonidet P-40, 0.5% nordeoxycholate, 1 mM EDTA acid, 150 mM NaCl, 50 mM Tris base, pH 8.3, 0.02% NaN_3 , and bovine serum albumin 1 mg/ml) for 30 min and filtered through 0.22 μm Millex-GS filters (Millipore SA). Lysates were precleared using *Staphylococcus aureus*. Immune complexes were formed with unconcentrated CAM 5.2 supernatant overnight at 4°C. Monoclonal antibody W6/32, which binds to monomorphic determinants of HLA-A, B, C molecules,¹² was used as a positive control. Immune complexes were precipitated using *Staph aureus*.¹³ Proteins were separated on a 10% (wt/vol) polyacrylamide slab gel using the buffer system of Laemmli,¹⁴ stained with Coomassie brilliant blue, destained, dried, and exposed to X-Omat S x ray film (Kodak) at -70°C . ^{14}C -labelled molecular weight markers (Amersham International plc) were

used as comparative standards.

^{35}S -methionine (Amersham International plc) biosynthetically labelled SW837, SW620, SW48, and HT29 cells (Rudd *et al.*, unpublished observations) were treated in a similar manner; the monoclonal antibody LE61¹⁵ recognising cytokeratin proteins served as a positive control. Exposure was on SB-5 x ray film (Kodak) at -70°C .

IMMUNOPEROXIDASE REACTION ON FROZEN TISSUE SECTIONS

Frozen tissue sections were cut and stained as outlined above.

IMMUNOPEROXIDASE REACTION ON FIXED, PARAFFIN EMBEDDED TISSUES

Tissue sections were dewaxed and taken through graded alcohols to water. Endogenous peroxidase was inhibited with freshly prepared 0.5% hydrogen peroxide in methanol for 10 min at room temperature. The sections were digested with trypsin (0.1% trypsin (Sigma type II), 0.1% CaCl_2 , pH 7.8, with NaOH) for 10 min in a humid chamber at 37°C .¹⁶ The method then proceeded as for the frozen tissue sections. CAM 5.2 supernatant was applied to the sections followed by peroxidase conjugated rabbit antimouse immunoglobulin. The peroxidase reaction was developed using diaminobenzidine and hydrogen peroxide. Washes between each stage were with TBS. Sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted.

IMMUNOFLUORESCENCE ON FRESH TISSUE

Frozen sections were prepared as previously outlined and fixed in acetone for 5 min. Unconcentrated CAM 5.2 supernatant was applied for 1 h at room temperature. This was followed by rabbit antimouse immunoglobulin conjugated FITC (Miles), diluted 1/40 in tissue culture medium, which was applied for 45 min at room temperature. Washes between each stage were with TBS. A final wash was given with distilled water before mounting the sections in Gelvatol 20/30 (gift of Monsanto, Springfield, Massachusetts, USA).

Results

One hundred and sixty colonies were screened by ELISA on the immunising cell line, of which 13 produced strongly positive results. Of these, eight survived transfer to larger cultures and were examined on freshly frozen human tissue sections. Four produced no reaction; three produced good generally positive staining; and one, CAM 5.2, an IgG2a immunoglobulin, was strikingly epithelial specific.

Table 1 CAM 5.2 staining of fixed normal adult tissues

Tissue type	Staining reaction
Skeletal muscle	—
Smooth muscle	±
Cartilage	—
Lymphoid tissues	—
Thymus-hassals corpuscles and epithelial cells	+
Liver	—
Most hepatocytes	—
Few adjacent to portal tract	+
Gall bladder and bile duct epithelium	++
Skin	—
Epidermis	—
Appendages (except sebaceous glands)	++
Breast	—
Ductal epithelium	++
Acinar epithelium	++
Gastric epithelium	++
Small bowel epithelium	++
Large bowel epithelium	++
Parathyroid epithelial cells	++
Thyroid epithelium	++
Adrenal glandular cells	—
Lung	—
Bronchial epithelium	++
Bronchial glands	++
Kidney	—
Proximal convoluted tubules	++
Distal convoluted tubules	++
Bowmans capsule cells	+
Ovary epithelial cells	++
Fallopian tube epithelium	++
Endometrium	—
Epithelium	++
Stroma	—
Cervix	—
Endocervical glands	++
Ectocervical epithelium	—
Testis—all cells	—
Epididymal tubules	++
Rete testis	++
Prostatic epithelium	++
Urothelium	++
Salivary glands	—
Ductal	++
Acinar	++
Placenta syncytio- and cytotrophoblast	++
Mesothelium	++

+ = positive, ++ = strongly positive, — = negative.

Three major bands at 50 000, 43 000, and 39 000 daltons were identified by the antibody on protein immunoblotting (Fig. 1), which suggested the antibody might be directed against the lower molecular weight cytokeratins and so not react with the cell surface. This was confirmed by the fact that no ¹²⁵I surface labelled material was precipitated by the antibody (unpublished observations) and that in preliminary studies it did not react with whole viable cells in immunofluorescence. CAM 5.2 did, however, precipitate similar bands to those seen on immunoblotting with ³⁵S-methionine labelled whole cell extracts (Fig. 2). Higher molecular weight cytokeratin polypeptides have not been identified on protein immunoblots using human foreskin keratinocytes (unpublished observations).

Immunofluorescence studies on sections of normal colon showed the characteristic filamentous,

Table 2 CAM 5.2 staining of formalin fixed tumours

Tumours staining positively	Number tested
Colorectal adenocarcinomas	15/15
Gastric adenocarcinomas	3/3
Breast adenocarcinomas	5/5
Lung adenocarcinomas	3/3
Prostatic adenocarcinomas	3/3
Ovarian adenocarcinomas	4/4
Renal adenocarcinomas	2/2
Hepatocellular carcinomas	2/2
Adenocarcinoma unspecified	7/7
Transitional carcinomas	2/2
Oat cell carcinomas	8/8
Carcinoids of appendix	6/6
Carcinoids of stomach	2/2
Squamous carcinoma of lung	2/2
Squamous carcinoma of floor of mouth	2/2
Squamous carcinoma of cervix	3/3
Squamous carcinoma of skin	2/2
Merkel cell carcinoma	1/1
Chorion epithelioma	2/2
Testicular teratoma, MTI and MTU	—
Most non-keratinising epithelium	2/2
Areas showing yolk sac differentiation	1/1
Pleomorphic adenoma	2/2
Carcinosarcoma—epithelial areas	2/2
Mesothelioma	—
Pleural	4/4
Peritoneal	1/1
Thymomas—epithelial	4/4
Anaplastic tumours unspecified	3/6

Tumours staining negatively	Number tested
Malignant melanoma	3/3
Carcinosarcoma—sarcoma cells	2/2
Lymphomata	10/10
Thymomas—lymphocytic	1/1
Ewing's sarcoma	2/2
Neuroblastoma	4/4
(two contained areas of rosetting and the cells comprising the rosettes were positive)	—
Other sarcomas	3/3
Malignant fibrous histiocytoma	3/3

cytoplasmic distribution of cytokeratins. Unfortunately, although these intermediate filaments can be visualised under the light microscope, they do not photograph for the purpose of illustration as well as when the procedure is performed on fixed cell lines (Dr B Lane, personal communication).

The antibody reacted strongly and consistently with all epithelial tissues in preliminary tests on formalin fixed, paraffin embedded tissue sections. Trypsin digestion before the immunohistochemical reaction produced even staining comparable to that seen in freshly frozen tissue sections. In view of this (Fig. 3), the full range of reactivity was determined on normal and malignant human tissues (Tables 1 and 2) and freshly frozen fetal tissues of 20 weeks' gestation (Table 3).

CAM 5.2 stained all normal adult epithelia tested (Table 1), with the exception of stratified squamous

Table 3 CAM 5.2 staining of fresh tissues from a fetus of 20 weeks' gestation

Tissue type	Staining reaction
Cerebellum	—
Skeletal muscle	—
Smooth muscle	±
Cartilage	—
Heart muscle	—
Lymphoid tissues	—
Liver—hepatocytes	++
Skin	
All keratinocytes	+
Basal layer	++
Appendages	++
Oesophageal epithelium	++
Gastric epithelium	++
Small bowel epithelium	++
Large bowel epithelium	++
Peritoneal mesothelial cells	++
Pituitary—all anterior cells	++
Parathyroid gland epithelium	++
Thyroid epithelium	++
Pancreas	
Ductal cells	++
Exocrine epithelial	++
Endocrine cells	++
Adrenal—peripheral glandular cells	+
Lung	
Bronchial lining cells	++
Alveolar lining cells	++
Kidney	
Proximal convoluted tubules	++
Distal convoluted tubules	++
Collecting tubules	++
Bowman's capsule cells	++
Ovary	
Ductal structures in mesovarium	++
75% of sex cord cells	++

+ = positive, ++ = strongly positive, — = negative.

epithelia. The skin appendages reacted positively, including a single basal layer of epithelial cells towards the lower end of the hair shaft. The only exception to this were the sebaceous glands, which were negative. Despite the negative reaction on squamous epithelium, all of the squamous carcinomas, regardless of site of origin that we have studied have been positive (Table 2). Some differences were found between the results in adult and fetal tissues. Fetal hepatocytes stained uniformly positively, whereas in adults only a few hepatocytes adjacent to portal tracts stained lightly positively. In this context it is of interest to note that all the hepatomas studied were strongly positive. The other differences in reactivity between fetal and adult normal tissues were on the adrenal gland. In the fetus the cells immediately beneath the capsule were positive whereas in the adult no positive cells were noted. All carcinomas arising from neuroendocrine cells studied were positive. Mesothelium and reticular epithelia of the thymus stained positively, as did tumours arising from these tissues.

There was pale cytoplasmic staining of smooth muscle in some sections. Similar cross reactivity has been noted with HMFG1 and other cytokeratin

antibodies (Dr EB Lane and Dr J Taylor-Papadimitriou, personal communication).

Discussion

A monoclonal antibody, CAM 5.2, which is an IgG2a immunoglobulin, has been isolated. The antibody defines the intracellular low molecular weight cytokeratin polypeptides of about 50 000, 43 000, and 39 000 daltons. On formalin fixed paraffin embedded tissue sections CAM 5.2 consistently stains normal adult epithelial cells with the exception of stratified squamous epithelia. All tumours arising from secretory epithelia and all squamous carcinomas that we have examined also stain with CAM 5.2.

Nineteen different cytokeratin polypeptides have been identified by two dimensional gel electrophoresis of intermediate filaments from different human sources; the three identified by CAM 5.2 correspond to 8, 18, and 19 in Moll's catalog.² No epithelial tissue or cell line has been found to contain only one cytokeratin species, and the complexity of patterns varies in different epithelia. In epithelia derived from the gastrointestinal tract the cytokeratin proteins are of lower molecular weight, similar to those expressed during early embryogenesis, whereas the higher molecular weight components are found in the more complex epithelia such as the stratum corneum of the epidermis. Metastases of solid tumours retain the intermediate filament type of their primary tumour, which is of some importance when the heterogeneity of malignant cells within a single human cancer is considered.¹⁷

The non-selective staining of epithelial tumours makes CAM 5.2 a more reliable marker for differentiating carcinoma from lymphoma, melanoma, and sarcoma, and in screening effusions and bone marrows for carcinoma cells of any type (Fig. 3d), than previously described epithelial markers such as HMFG1 and 2^{18 19} and EMA.²⁰ These latter markers are more selective in their epithelial reactions, and within tumours they show more heterogeneity of staining than CAM 5.2. These two attributes probably make them more useful tools for studies on individual tumour types than CAM 5.2, but their selectivity is a disadvantage in the situations already mentioned. Other monoclonal antibodies raised against the cytokeratins have not reacted reliably on formalin fixed paraffin embedded material.^{1 3 4}

The positive reaction of CAM 5.2 with tumours of neuroendocrine origin^{22 23} is an interesting observation which requires further study. This finding is in agreement with that of Ramaekers *et al*⁴ with their antikeratin antibody RGE53.

The difference in staining reaction between stratified squamous epithelia and squamous carcinomas may prove useful as a prognostic indicator in premalignant and early malignant lesions arising in these tissues.

In conclusion, we emphasise the use of monoclonal antibodies, rather than conventional antisera, to intermediate filaments in routine surgical pathology. Antibodies such as CAM 5.2, which recognise different epitopes on the cytokeratin proteins and produce consistent results on formalin fixed paraffin embedded tissue, allow the clinical pathologist to distinguish with confidence anaplastic carcinomas from lymphomas, melanomas, and sarcomas. In addition, single or small numbers of epithelial cells can be easily identified in abnormal situations, such as bone marrow aspirates, cerebrospinal fluid aspirates, malignant effusions, and lymph nodes.

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