Immunohistochemical detection of Ca antigen in normal, dysplastic and neoplastic squamous epithelia of the human uterine cervix

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SUMMARY Immunohistochemical staining was performed on biopsies and cytological samples from normal, dysplastic and neoplastic squamous epithelia using the monoclonal Ca 1 antibody. The results of staining 92 biopsies and 20 cytological samples are described and it is reported that positive staining with Ca 1 antibody was detected in normal, dysplastic and neoplastic epithelia.

The role of the Ca 1 antibody in the study of cervical cancer is discussed.

One of the most important questions of cancer immunology is whether human tumour cells express antigens which are not expressed by normal cells. An antigen described by Ashall, Bramwell and Harris¹, and designated the Ca antigen, was found on the cell surface in a wide range of malignant human cell lines, but not in non-malignant cell strains. The antigen was detected by means of a monoclonal IgM antibody, the Ca 1 antibody. Following on from this discovery McGee *et al*² used the Ca 1 antibody in an immunohistochemical procedure to detect the Ca antigen in tissue sections. These workers showed that the majority of malignant tumours examined expressed the Ca antigen, whereas the only normal tissues to bind the Ca 1 antibody were the transitional epithelium of the urinary tract and the epithelium of the fallopian tube.

In the present study the monoclonal Ca 1 antibody was used in immunofluorescence and immunoperoxidase procedures on fresh and fixed tissue from the cervix uteri. Carcinoma of the cervix uteri is exceptional in that preinvasive conditions can be diagnosed cytologically and can be readily biopsied. Thus biopsies and cytological samples from normal, dysplastic and invasive epithelia were examined for the presence of the Ca antigen in an attempt to investigate the relation between malignancy and the occurrence of the Ca antigen.

Material and methods

IMMUNOHISTOLOGICAL REAGENTS

The following antibodies were obtained from Wellcome Diagnostics, Temple Hill, Dartford, Kent: monoclonal Ca 1 antibody (code RP82); monoclonal IgM antibodies to meningococcal bacteria (code MB34, MB36) selected for use as controls in order to demonstrate the specificity of the Ca 1 antibody; and peroxidase conjugated rabbit antimouse Ig antibody.

Fluorescein isothiocyanate (FITC) conjugated goat antimouse IgM antibody was obtained from TCS, 10 Henry Road, Slough, Bucks. Diaminobenzidine tetrahydrochloride and trypsin were obtained from Sigma Chemical Co, Fancy Road, Poole, Dorset.

TISSUES

Most of the specimens were of fresh frozen tissue collected by biopsy from a referral colposcopy clinic at Dulwich Hospital. Cervical scrapes were also collected at this clinic. Other fresh cervical tissue was obtained from hysterectomy or cone biopsy specimens at King's College Hospital or Dulwich Hospital. Normal skin, normal colon, and colon tumour tissues were also collected for use as controls. All fresh tissue was snap frozen in liquid nitrogen within one hour of collection, and stored at -80°C prior to cryostat sectioning.

Paraffin embedded material was also collected.

TISSUE SECTION PREPARATION

Fresh frozen tissue stored at -80°C was sectioned at $5\text{ }\mu\text{m}$ in a cryostat. The sections were air dried for 30 min and stained immediately using the immunofluorescence procedure (below).

Sections ($5\text{ }\mu\text{m}$) were prepared from cervical biopsies or intact cervixes which had been fixed in neutral buffered formalin for $>24\text{ h}$ and routinely processed to paraffin wax. The sections were mounted on glass slides, dried at 37°C , dewaxed in xylene, rehydrated in alcohols and washed in distilled water prior to immunoperoxidase staining (below).

CELL PREPARATION

A single cervical scrape was taken at the colposcopy clinic. After preparing a smear for routine cytological evaluation the scrapings were suspended in 20 ml of phosphate buffered saline, pH 7.2 (PBS) used here as a holding solution. The suspended cells were processed for immunohistochemistry within three hours of collection. The suspended cells were washed twice in PBS and the cell density adjusted to approximately 100 000 cells/ml; 0.25 ml of suspension was dropped into each chamber of a 12 chamber cytospin (Shandon) and after a 5 min spin at 700 rpm, the prepared slides were removed and fixed without drying in 50:50 (chloroform:acetone) for 5 min. The slides thus prepared were stored at -80°C prior to immunostaining. The technique used bears similarities to that used on cervical cell imprints³ rather than to that used on unfixed cervical cells in suspension.^{4,5}

IMMUNOPEROXIDASE METHOD

The immunoperoxidase procedure used was based on that used by McGee *et al.*² Sections and cell preparations were incubated with the Ca 1 antibody or the control antibodies (MB34, MB36) for 60 min. The antibodies were diluted 1/12 in a solution composed of 10% fetal calf serum (FCS) and 10% bovine serum albumin (BSA) in PBS, pH 7.4. The sections were washed three times for 10 min in PBS and incubated for 30 min in rabbit antimouse IgM peroxidase conjugate (Wellcome) at a dilution of 1/50 in PBS containing 10% FCS, 10% BSA and 5% human AB serum. The sections were washed three times for 10 min in PBS and reacted for 5 min with diaminobenzidine tetrahydrochloride at $500\text{ }\mu\text{g/ml}$ in PBS containing 0.01% (vol/vol) hydrogen peroxide (H_2O_2). The sections were washed in tap water for 5 min, stained with haematoxylin, dehydrated in alcohols, cleared in xylene and mounted in DPX (BDH Chemicals, Poole, Dorset).

To remove endogenous peroxidase the sections and cell preparations were preincubated with 0.5%

H_2O_2 in methanol for 10 min. To remove any "non-specific binding", as described by McGee *et al.*,² samples of the sections and cell preparations were incubated for 30 min with 0.5% (wt/vol) trypsin in PBS.

IMMUNOFLUORESCENCE

Sections of tissue were incubated for 60 min with Ca 1 or control IgM diluted 1/12 in PBS. The sections were washed three times for 10 min in PBS and incubated for 30 min in goat antimouse/FITC conjugate (Tago) at a dilution of 1/50 in PBS. The sections were then mounted in glycerol/PBS and examined using a Reichert-Jung Polyvar microscope with a 200 W mercury vapour lamp, a blue BP 455-490 incident light exciter filter, a DS 500 dichroic mirror and a blue LP 515 barrier filter.

Results

USE OF CONTROL ANTISERA

The result of using the monoclonal antibodies MB34 and MB36 on cervical tissue in both of the immunochemical techniques showed that there was a complete absence of staining. For this reason results obtained using the Ca 1 antibody were not attributed to non-specific binding.

USE OF CONTROL TISSUES

The normal colon and normal skin tissue did not stain with the Ca 1 antibody. A weak focal reaction was found in sections of colon tumour.

COMPARISON OF FIXED AND FRESH TISSUE

Studies comparing immunohistochemistry on frozen and paraffin embedded sections from adjacent blocks showed similar staining patterns when stained with Ca 1 antibody. From this it was deduced that the Ca antigen was not destroyed by fixation or processing.

COMPARISON OF IMMUNOFLUORESCENCE AND IMMUNOPEROXIDASE

Comparison of immunofluorescence and immunoperoxidase on serial sections of cervical tissues showed similar staining patterns. Therefore the results obtained using both techniques were combined.

USE OF TRYPSIN

Trypsin incubation as a pretreatment for the immunoperoxidase method did not alter the patterns of staining. The observed staining patterns were considered to be due to the presence of the Ca antigen and not to the occurrence of "non-specific binding" which could be removed with trypsin.²

Table 1 Immunohistochemical staining with Ca 1 antibody of biopsies from the cervical transformation zone

Histological diagnosis	No of biopsies examined	No staining with Ca 1 antibody
Normal mature and immature metaplastic squamous epithelium	27	25
Mildly dysplastic epithelium	15	15
Moderately dysplastic epithelium	22	22
Severely dysplastic epithelium	8	8
Carcinoma in situ of epithelium	8	8
Invasive carcinoma of epithelium	15*	15
Total	95	93

*Fourteen of these biopsies were paraffin embedded and stained by the immunoperoxidase technique.

Fig. 1 Cervical biopsies labelled with the immunoperoxidase method and counterstained with Mayer's haematoxylin, showing positive staining with the monoclonal Ca 1 antibody. (a) normal squamous epithelium showing staining (arrow) $\times 200$. (b) severely dysplastic epithelium showing focal staining only (arrow) $\times 225$. (c) poorly differentiated invasive squamous cell carcinoma showing focal staining (arrow) $\times 225$.

Fig. 2 Invasive squamous cell carcinoma labelled with the immunoperoxidase method and counterstained with Mayer's haematoxylin showing positive staining with the monoclonal Ca 1 antibody. (a) Staining predominantly on cell membranes (arrow) $\times 225$. (b) Staining predominantly in the cytoplasm (arrow) $\times 525$.

CERVICAL BIOPSIES

Sections from 81 fresh biopsies were stained by the immunofluorescence technique and sections from 14 fixed biopsies were stained by the immunoperoxidase method. Immunohistochemical staining with the Ca 1 antibody was seen in almost all the material examined (Table 1). This staining was seen in normal, dysplastic and invasive epithelia (Fig. 1). The staining pattern was uneven. In some areas all the cells of the epithelium were intensely stained and in other areas only isolated cells or groups of cells were stained.

Although this variation in staining was found in representatives from all the histological groups, there was a tendency for normal and metaplastic epithelia to exhibit least widespread staining. In the majority of sections the variation in histochemical localisation did not correlate with a variation in histopathology. Exceptions to this general observation were seen in a small number of cases in which staining coincided with the appearance of an area of dysplasia.

In almost all sections of dysplastic and normal epithelia the basal layer did not stain with the Ca 1 antibody. This distinction was not seen in invasive epithelia or in four sections showing carcinoma *in situ* or in four sections showing severe dysplasia.

The distribution of the Ca antigen within the cells was variable. The antigen was generally detected both in the cytoplasm and on the cell membrane, although some cells were apparently stained either in the cytoplasm or on the membrane (Fig. 2). Both the cytological distribution and the intensity of staining of the Ca 1 antibody varied within each section.

CELL PREPARATION

The results of the immunoperoxidase staining of the cell preparations showed positive staining in all but two of the 20 samples examined (Table 2). The normal cell preparations contained a number of positively stained cells. The dyskaryotic cell preparations contained a proportion of both positive normal cells and positive dyskaryotic cells. Similarly malignant cell preparations contained proportions of both positive normal cells and positive malignant cells. Examples of normal and dyskaryotic cells demonstrating positive membrane staining are shown in Fig. 3.

DISCUSSION

Histological and cytological samples of normal dysplastic and neoplastic cervical epithelia examined in this study showed expression of the Ca antigen. These results are at variance with those obtained by McGee *et al*² who found staining in squamous cell carcinoma of the cervix but not in normal cervical

Table 2 Immunoperoxidase staining with Ca 1 antibody of cervical cell preparations

Cytological diagnosis	No examined	No staining with Ca 1 antibody
Normal	4	3
Mild dyskaryosis	4	4
Moderate dyskaryosis	4	3
Severe dyskaryosis	4	4
Malignant	4	4
Total	20	18

Fig. 3 Cell preparations labelled with the Ca 1 antibody, using the immunoperoxidase method and counterstained with Mayer's haematoxylin showing positive membrane staining. (a) Normal squamous cells showing positive staining (arrow) $\times 525$. (b) Dyskaryotic squamous cells showing positive staining (arrow) $\times 525$.

epithelia. These workers do, however, report the occurrence of non-specific binding in the cervical epithelium. This binding was seen when using control monoclonal IgM antibodies, and could be removed by incubating the sections with trypsin. In the present study there was no non-specific reaction between cervical epithelium tissue and control IgM antibody. The binding of the Ca 1 antibody was not removed with trypsin, and was considered to be due to the presence of the Ca antigen. The pattern of staining found in colon tumour was similar to that reported by McGee *et al.*²

If expression of the Ca antigen was predominantly due to malignant or premalignant changes within the cells of the cervical epithelium the observed staining might be expected to occur in abnormal cells only. However, the variety of staining pattern seen in representatives of all the morphological groups suggests that in the cervical epithelium the expression of the Ca antigen is not related directly to malignancy. Some link with malignancy is suggested by those cases in which the occurrence of the antigen corresponded to the localised presence of dysplasia.

The observation of heterogeneity of staining seen here corroborates observations made by McGee *et al.*² who observed variation of staining within single tumours and cited an example of a malignant carcinoid in which the Ca antigen was detected in only two of three blocks. The distribution of this antigen is clearly not related in a simple way to the presence or absence of malignancy.

The absence of immunohistochemical staining in the basal layers of the squamous epithelium is difficult to explain. The apparent absence of the Ca antigen in such a layer of proliferating cells may have some significance in a study of the function of the Ca antigen, but at present this is not clear.

The results reported in this study suggest that the Ca 1 antibody does not have a significant role in the primary diagnosis or follow up of dysplastic and neoplastic lesions of the uterine cervix.

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