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Nuclear proteins of the bovine esophageal epithelium

I. Monoclonal antibody W2 specifically reacts with condensed nuclei of differentiated superficial cells

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SUMMARY

Cells from three layers of the bovine esophageal epithelium, representing different stages of differentiation, were dissociated and separated by Percoll gradient centrifugation into fractions of small, medium and large sizes. A majority of the large cells possessed condensed nuclei, a characteristic feature of terminal differentiation of the superficial epithelium. The small cells resembled the proliferate cells of the basal layer. In vitro culture of the esophageal epithelial cells resulted in proliferation of the small cells, colony formation, and, in some cases, differentiation into cells with condensed nuclei. Nuclei, or nuclear subfractions derived from cells of the different layers, were used as immunogens for the generation of hybridomas secreting monoclonal antibodies that bound specifically to different regions of the esophageal tissue. One such antibody, designated W2, labeled the condensed nuclei from the superficial layer of stratified esophageal and corneal epithelia in situ, as well as the large cells from esophageal culture in vitro. Thus, the expression of the W2 antigen may be associated with the process of nuclear condensation during epithelial differentiation. Immunoisolation of the target antigen of W2 from extracts of large cells of the bovine esophagus yielded a band of M_r ~33,000 on nonreducing polyacrylamide gels. This band dissociated into two polypeptides, of M_r ~22,000 and ~11,000, upon treatment with dithiothreitol. Amino acid sequence analysis of the larger polypeptide showed extensive homology to a group of small calcium-binding proteins, including two helix-turn-helix motifs designated as the EF-hand, characteristic of the configuration of the metal-ion coordinating ligands of the calcium-binding site. Similarly, the sequence at the amino terminus of the polypeptide of ~11,000 indicated that it was the light chain counterpart of the same calcium-binding protein complex.

Key words: calcium binding protein, condensed nuclei, epithelial cell differentiation

INTRODUCTION

Molecules of the cell nucleus and their unique patterns of expression undoubtedly play an important role during development and cellular differentiation. One example is the histones, of which a particular subtype or variant is expressed during the maturation of specific cell types (Stein et al., 1984). Histone H5, possibly an extreme variant of H1, is found in the nucleated erythrocytes of birds, fish, reptiles, and amphibians (Smith et al., 1980; Tsai and Hnilien, 1975). The expression of this tissue-specific gene product occurs in cells of a developmentally unique maturation pathway, in which their nuclei are condensed and ultimately eliminated. In contrast to the large body of data on the histone variants, much less is known about other nuclear proteins which may play important roles in the differentiation program.

Epithelia that show stratification are particularly amenable for analysis of components that are specific for various stages of differentiation. The esophageal epithelia, for example, are thick, and progressive changes at different stages of differentiation, such as nuclear condensation, can readily be observed. The composition, lineage, morphology and growth features of the cells within the stratified epithelia have been defined (Green et al., 1982). The epithelial cells can be grown in culture and attempts to manipulate the differentiation program in vitro can be made (Doram et al., 1980). There are also well-documented changes in cytoplasmic proteins during the differentiation stages of the stratified epithelium. For example, it has been demonstrated that expression of the keratins, a family of intermediate filament proteins present in almost all epithelial tissue, follows well-defined “rules” that depend on cell type, cellular growth environment, stage of development

and differentiation, and disease state (Eichner et al., 1984). These detailed analyses have provided much insight into the different functional roles that the various keratin polypeptides may play during epithelial differentiation. Finally, large amounts of tissue can be isolated and, therefore, sufficient starting material is available for biochemical analysis.

Our approach to describing the unique spectrum of nuclear proteins specific for different stages of esophageal differentiation is to raise monoclonal antibodies directed against these proteins. We have undertaken this program, using the bovine esophageal epithelium (BEE) as our source of immunogen and target of analysis. In the present paper, first in the series, we describe in detail the cells of the BEE system, the immunization and screening protocols and the analysis of a monoclonal antibody (mAb) specific for condensed nuclei of terminally differentiated cells.

MATERIALS AND METHODS

Isolation of epithelial cells from bovine esophageal epithelium (BEE)

Routinely, bovine esophagus from four freshly slaughtered animals was used for each preparation. After washing with phosphate-buffered saline (PBS) containing 2 mg/ml penicillin, 2 mg/ml streptomycin, 16 mg/ml gentamycin, and 1.25 μg/ml fungizone, the esophagus was cut open and the smooth muscle layer, the connective tissue and the blood vessels underneath were removed. The remaining epithelium was minced into small pieces and washed with 10 volumes of Tris-buffered saline, and filtered through one layer of cheesecloth. The filtrates were immediately subjected to centrifugation (1,500 ***g***, 5 min) for the isolation of large, cornified cells. The cell pellets were further suspended in 10% Percoll in TKM buffer (20 mM Tris, 5 mM KCl, 1 mM $MgCl_2$, pH 7.2) and centrifuged (4,000 ***g***, 1 min). The supernatant was diluted five-fold with TKM and centrifuged (1,500 ***g***, 5 min) again. This yielded the large cell fraction. Alternatively, the large cells can be obtained directly by scraping the epithelium with a dissecting knife.

To isolate small and intermediate-size cells, the minced epithelial tissue was treated with 0.3% proteases (Type XIV from *Streptomyces griseus*, Sigma) in PBS for two h at 37°C, along with vigorous shaking. The mixture was filtered through four layers of cheesecloth and was layered on top of 75% Percoll (in TKM) and centrifuged (2,000 ***g***, 5 min) to remove large cell clumps and particles. The supernatant was collected, diluted five-fold with TKM and centrifuged (2,000 ***g***, 5 min) to pellet the cells. After resuspension in 40% Percoll, the cells were centrifuged (2,000 ***g***, 10 min); the medium cells remained in the supernatant, whereas the pellet contained the small cells. The supernatant was diluted five-fold and centrifuged (2,000 ***g***, 5 min); the pellet from this step was resuspended in 20% Percoll and centrifuged (2,000 ***g***, 10 min) to remove any contaminating large cells in the supernatant. The medium cells were recovered in the pellet and washed one more time with TKM buffer.

Light microscopy and ultrastructural analysis were carried out according to the method described (Quaroni et al., 1979) to compare the isolated cells with those in the BEE tissue. For electron microscopy, thin sections were examined in a JEOL T330 electron microscope.

Immunization and generation of hybridoma

Large cells, isolated by scraping the esophageal mucosa, served as the starting point for our immunogen preparation for hybridoma-secreting antibodies reactive against the nuclei in the superficial layer. The large cells were extracted with 2 M NaCl. The total cellular extract was then dialyzed against water. After centrifugation (10,000 ***g***, 10 min), the resultant supernatant fraction was used as immunogen. It was mixed with an equal volume of complete Freund's adjuvant, and injected subcutaneously as well as intraperitoneally into Balb/c mice (0.2-0.4 ml/mouse). After two weeks, booster injections of the immunogen in incomplete Freund's adjuvant were given at bi-weekly intervals. Three days before killing the mouse for spleen cell isolation and fusion, a final booster injection of the immunogen was given intravenously.

The protocols for spleen cell isolation, fusion with PAI myeloma cells (a subclone of the NS1 strain), selection and culture of hybridoma have all been previously described (Galfre and Milstein, 1981; Kohler and Milstein, 1975). The hybridoma cultures were screened for nuclear-specific antibodies by immunofluorescence on a "sandwich" of frozen sections of various bovine tissues, including esophagus, cornea, epidermis and liver. Tissue samples were cut into pieces of approximately 10 mm^2 in size and 2 mm in thickness and soaked in Dulbecco's modified Eagle's medium. Individual pieces from different tissues were layered on top of one another in an aluminum foil cup approximately 10 mm in diameter. These tissue layers were fixed with the embedding medium OCT (Miles Scientific), immersed in isopentane and frozen in liquid nitrogen. The tissue samples were sectioned in a Reichart microtome. The sections collected on a glass slide were fixed in methanol/acetone (1:1, v/v) at −20°C, and then stored at −80°C.

For screening hybridoma cultures, the sections were incubated with supernatants of the various cultures for 1 h at room temperature. The binding of mouse immunoglobulin to tissue sections was detected by fluorescein-conjugated, goat anti-mouse immunoglobulin (Cappel Laboratory). The concentration of this secondary antibody was titered (usually 1:100 dilution of the commercial stock) such that in parallel control incubations of tissue samples in the absence of primary antibody, little or no fluorescence was detected. The incubation was carried out for 30 min at room temperature; included in the incubation was the DNA-specific dye, bis(benzimidazole)Hoechst 33258 (Cesarone et al., 1979) (Sigma; 5 μg/ml). After each incubation, the samples were washed three times for 5 min each in PBS. Immunofluorescence slides were viewed with a Zeiss microscope with the appropriate filters for fluorescein or Hoechst dye fluorescence.

In double immunofluorescence experiments, fixed tissue sections of BEE were first incubated with ascites fluid of hybridoma W2 (1:1000 dilution) at 37°C for 1 h. After washing with PBS three times, the same sections were incubated for another hour at 37°C with polyclonal rabbit antiserum against BEE22 (1:1000 dilution) or with polyclonal rabbit antiserum against BEE11 (1:50 dilution). The washing with PBS was then repeated three times. The sections were then incubated with a mixture of fluorescein-conjugated, goat anti-mouse immunoglobulin (Cappel) and rhodamine-conjugated, goat anti-rabbit immunoglobulin (Vector) for fluorescence microscopy.

Ascites fluids and polyclonal antisera

mAb W2 was collected in large amounts from ascites fluid of mice injected with hybridoma W2 cells. Balb/c mice were first injected with 1 ml of pristane (2,6,10,14-tetramethyl pentadecane, Sigma) into the intraperitoneal cavity (Bartal and Hirshaut, 1987). After 7-10 days, hybridoma W2 cells suspended in PBS (10^7 cells/ml) were injected into the same cavity (0.5 ml/mouse). The ascites fluid was collected from the peritoneal cavity by aspiration

through an 18 guage needle, centrifuged (2,000 ***g***, 10-15 min), and the supernatant was divided into samples for storage (−20ºC).

As a control in some of the experiments using ascites fluid containing mAb W2, we have carried out, in parallel, experiments using ascites fluid from an irrelevant hybridoma M2. This hybridoma, a generous gift of Dr Pan-Chyr Yang (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan), was generated by immunization of mice with a tumor cell derived from bronchial epithelium and was directed against a cell surface component.

Polyclonal antisera directed against polypeptides BEE22 and BEE11 were raised in rabbits. The polypeptides were eluted from gel slices after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) as described (Hager and Burgess, 1980). After concentration, the samples were mixed with complete Freund's adjuvant and injected into rabbits subcutaneously. After 10 days, the rabbits were injected at the same sites with the immunogen mixed in incomplete Freund's adjuvant. The rabbits were bled 10 days after the second injection, as well as after all subsequent boosters.

Cell culture

Several cultured cell lines were used in the present study: (1) human esophageal carcinoma cell lines TE-9 and CE-48 (Hu et al., 1984), (2) human glioma cell line U-251 (Wang et al., 1984), and (3) mouse fibroblast line 3T3 (Todaro and Green, 1963). All cell lines were cultured in Dulbecco's modified Eagle's medium containing 99 i.u./ml of penicillin, 75 i.u./ml of streptomycin and 10% fetal calf serum (GIBCO).

For in vitro culture of BEE cells, we followed procedures previously described for the culture of epithelial cells (Green et al., 1979; Quaroni et al., 1979). Briefly, the tissue was washed with PBS containing 2 mg/ml penicillin, 2 mg/ml streptomycin, 16 mg/ml gentamycin and 1.25 μg/ml fungizone (GIBCO). The tissue was cut into small pieces, rinsed with Dulbecco's modified Eagle's medium, digested with 30 ml of Dispase (Boehringer-Mannheim; 2.4 i.u./ml) at 4ºC overnight (or 37ºC for 1-2 h). The Dispase was then neutralized by the addition of 3 ml of calf serum. The remaining pieces of tissue were further dissociated with 0.25% trypsin, 0.017% EDTA in PBS (~40 ml) at 37ºC for 45-90 min. Trypsin activity was neutralized by the addition of 4 ml of calf serum. The dissociated tissue was allowed to stand for 10 min; the supernatant was then centrifuged (1,000 ***g***, 8 min). The pellet was resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.4 μg/ml hydrocortisone, mixed with feeder layer 3T3 fibroblasts that had been pretreated with mitomycin C (4 μg/ml) and then plated into culture dishes (Rheinwald and Green, 1975). Colonies of cells were revealed with Wright stain (Sigma).

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to the procedure of Laemmli (1970), using 17.5% acrylamide in the running gel, both in the presence and absence of dithiothreitol (DTT). The separated proteins were revealed by Coomassie blue staining (Holbrook and Leaver, 1976). Alternatively, the separated proteins were transferred onto nitrocellulose paper (Towbin et al., 1979) and subjected to western blotting with specific antibodies. The primary antibody, from either ascites fluid of mouse hybridoma (1:500 dilution) or polyclonal rabbit antiserum (1:5000 dilution), was incubated overnight at 4ºC with the nitrocellulose strips. The secondary antibody, either peroxidase-conjugated, goat anti-mouse immunoglobulin (1:1000 dilution; Sigma) or peroxidase-conjugated, goat anti-rabbit immunoglobulin (1:1000 dilution; Vector), was incubated at room temperature for 1 h.

Immunoisolation of the W2 antigen and sequence analysis

A 2 M NaCl extract of large cells of BEE, prepared in the same way as the original immunogen fraction, was dialyzed extensively against PBS and then centrifuged at 10,000 ***g*** to remove particulate material. The supernatant fraction (3 ml) was incubated with 150 μl of normal rabbit serum for 1 h at 4ºC and then passed through a column (V_T = 1 ml) of Protein A-Sepharose (Sigma). This step removes material that binds nonspecifically to immunoglobulins and Protein A in the immunoprecipitation procedure. The flow-through fraction of the initial Protein A-Sepharose column was then incubated with W2 ascites fluid at 4ºC for 12 h. The mixture was then incubated with Protein A-Sepharose beads for 1 h on ice. The material bound on the beads was washed by centrifugation and resuspension in PBS and then subjected to SDS-PAGE.

Alternatively, mAb W2 was covalently coupled to Affi-Gel 10 (BioRad). A 2 M NaCl extract of large BEE cells was dialyzed against PBS and then passed through the affinity column containing mAb W2. The bound material was eluted with 0.1 M glycine-HCl, pH 3.0.

Polypeptide BEE22 was eluted from SDS gels (Hager and Burgess, 1980) and digested (at an approximate molar substrate-to-enzyme ratio of 50-to-1) with the following enzymes: (1) trypsin (Boehringer-Mannheim), 0.1 M Tris, pH 8.5, 4 h, 37ºC; (2) clostripain (endoproteinase Arg-C; Promega), 50 mM Tris, pH 8, 2 mM DTT, 1 mM $CaCl_2$, 18 h, 30ºC; (3) *Staphylococcus aureus* V-8 protease (endoproteinase Glu-C; Boehringer-Mannheim), 25 mM ammonium carbonate, pH 7.8, 18 h, 25ºC. The digestion mixture was fractionated by high pressure liquid chromatography (HPLC) on a C_{18} μBondapak column (3.9 × 300 mm) using 0.1% trifluoroacetic acid/H_2O as the solvent. The column was developed in 0.08% trifluoroacetic acid/acetonitrile, using a 0%-80% gradient and a flow rate of 1 ml per min. Isolated peptides were subjected to amino acid sequence analysis in an Applied Biosystems 477A gas phase sequencer. Polypeptide BEE11, after SDS/PAGE, was directly transferred to an Immobilon-P membrane (Millipore) and subjected for direct amino acid sequencing by an Applied Biosystems 477A sequencer.

RESULTS

Fractionation and characterization of the cells of the bovine esophageal epithelium

The bovine esophagus is a stratified tissue in which cells of different morphology are segregated into distinct layers, observable by light microscopy of frozen sections (Fig. 1A). Ultrastructural analysis showed that the superficial layer consisted mainly of large cells, with a distinctive condensed nucleus and a high ratio of cytoplasm-to-nucleus (Fig. 1B-a). In contrast, the basal layer contained small cells with a low cytoplasm-to-nucleus ratio (Fig. 1B-c), while the middle suprabasal layer contained a mixture of cells, heterogeneous in size, morphology, and nuclear-to-cytoplasmic ratio (Fig. 1B-b).

When the bovine esophageal tissue was digested with protease to dissociate the component cells, three main cell types, arbitrarily characterized by size, could be discerned. These are designated as large cells, medium cells and small cells. When a mixture containing these cell types was subjected to centrifugation on Percoll gradients, the three cell types were efficiently separated according to size. The large cell fraction was obtained with high purity (Table 1). Most

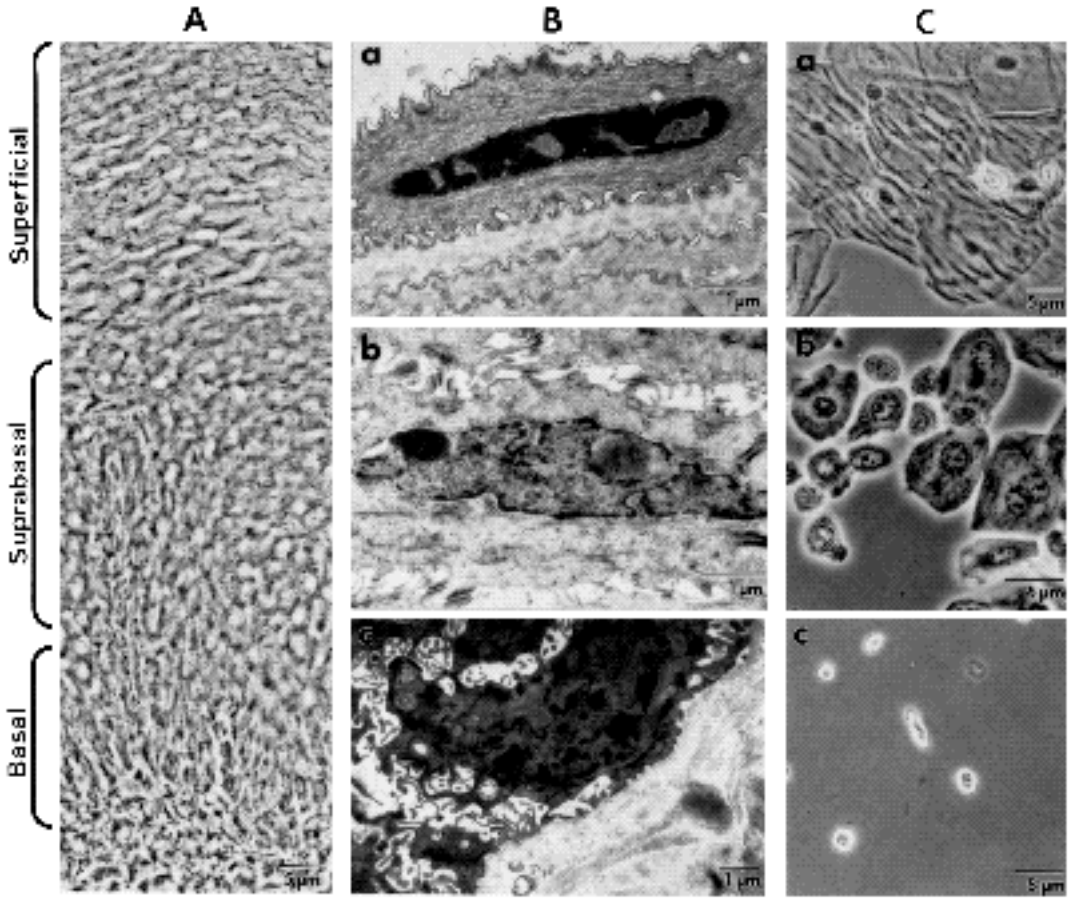

Fig. 1. The cells of the different layers of the bovine esophageal epithelium. (A) Phase contrast micrograph of a frozen section of the tissue (bar, 5 μm). (B) Electron micrographs of sections derived from the upper (superficial), middle (suprabasal) and lower (basal) layers (bar, 1 μm). (C) Phase contrast micrographs of dissociated cells isolated after Percoll gradient centrifugation (bar, 5 μm).

of the cells possessed very condensed nuclei, with a high ratio of cytoplasm-to-nucleus (Fig. 1C-a). A minor fraction of the isolated large cells contained no nuclei observable by light microscopy. The majority of the isolated large cells were resistant to treatment with 4% SDS and 10 mM DTT (Table 1), suggesting that the cells had been cornified. These large cells showed a distinct morphology, similar to the cells of the superficial layer when frozen sections of BEE were observed by light microscopy (Fig. 1A-a) or electron microscopy (Fig. 1B-a). Large cells can also be isolated in high yield simply by physical scraping of the esophageal mucosa, suggesting that they represented the cells of the outer surface layer.

Table 1. Three distinct cell populations isolated from bovine esophagus

	Large cells	Medium cells	Small cells
Cell diameter (μm)	15-25	3-7	≤2
Total cell number*	1.6×10^7	6.1×10^8	6.0×10^8
Purity (%)	100	78	87
Resistance to SDS and dithiothreitol†	87	22	13

The data reported represent a single isolation. Similar results were obtained in five different experiments.

*The yield of the various types of cells is based on the isolation procedure being carried out on esophagus derived from four animals.

†The concentrations of SDS and dithiothreitol used were 4% (v/v) and 10 mM, respectively.

The small cells were fairly refractile; their cytoplasm and nucleus cannot be distinguished easily because of the low cytoplasm-to-nucleus ratio (Fig. 1C-c). On the other hand, the medium cells were heterogeneous in size and shape; however, they all had a distinct, less condensed nucleus (Fig. 1C-b). On the basis of these features, we conclude that the large cells obtained from the Percoll fractionation were derived from the highly differentiated, superficial epithelium. By analogy, the medium cells and small cells were assigned to the suprabasal intermediate layer and the proliferative basal layer of BEE (Fig. 1), respectively.

Monoclonal antibodies reactive with condensed nuclei of superficial epithelium

For the generation of hybridoma, large cells were obtained by scraping of the esophageal mucosa. Total extracts (in SDS-PAGE sample buffer) of such cells showed prominent keratin polypeptides (*M*r ~44,000 and ~58,000), which constitute major protein components of this stratified epithelial tissue (Eichner et al., 1984). When these large cells were extracted instead with 2 M NaCl, the nuclei changed color; the untreated cells had dark nuclei, while the NaCl-extracted cells showed translucent nuclei. When the extract was dialyzed against water, the resulting supernatant fraction showed enrichment of polypeptides other than keratin. This material, which contained nuclear components, was used as the immunogen fraction for the generation of hybridoma W2.

Media derived from hybridoma lines were screened for nuclear-specific antibodies by immunofluorescence analysis on cryostat sections containing a sandwich of different bovine tissues: esophagus, cornea, epidermis and liver. The binding of the monoclonal antibodies was detected, in turn, by fluorescently-labeled, goat anti-mouse immunoglobulin. Included in this incubation was the DNA-specific dye Hoechst 33258. In all of these immunofluorescence studies, incubations containing no primary mouse immunoglobulins served as negative controls. Moreover, differences in the staining or lack of staining between different hybridoma supernatants also served as checks for the specificity of the mAb reactivity. From a thousand such screenings, several hybridomas secreting antibodies reactive with condensed nuclei were identified. Of these, clone W2 secreted the most striking and specific mAb; its properties are reported here in some detail.

mAb W2 stained the condensed nuclei of the superficial epithelial cells in esophagus, but not the cells at the basal stratum (Fig. 2). This was most strikingly apparent when the phase contrast micrograph (Fig. 2A-a) and that of nuclei stained by the DNA-specific dye Hoechst 33258 (Fig. 2A-c) were compared with the immunofluorescence photograph (Fig. 2A-b). Numerous nuclei that were revealed by the Hoechst dye failed to react with mAb W2. In cornea, the mAb W2 stained the nuclei of the superficial epithelium, but not the basal layer of the epithelium (Fig. 2B-b). The W2 antigen was not detected in epithelial cells of the limbal region, which is thought to consist of the stem cells for the corneal epithelium (Schermer et al., 1986). The W2 antigen was detected neither in the enucleated stratum corneum nor in the strata spinosum and basale of the epidermis (Fig. 2C-b). Finally, there was no staining of the nuclei of hepatocytes by mAb W2 (Fig. 2D-b). These results suggest that mAb W2 recognizes an antigen specifically associated with condensed nuclei of highly differentiated epithelial cells.

Using a different immunogen fraction, derived from nuclear components of small cells in the basal layer of BEE, we have generated other hybridoma such as W1 (Tang et al., 1993) and A1 (unpublished data). The staining patterns of mAb A1 on bovine esophagus, cornea, and epidermis were directly opposite to those observed with mAb W2. mAb A1 stained only the proliferative cells in the basal layer of these stratified epithelia, whereas mAb W1 stained the nuclei of all the cells studied, irrespective of species, tissue or stage of differentiation (Tang et al., 1993). Thus,

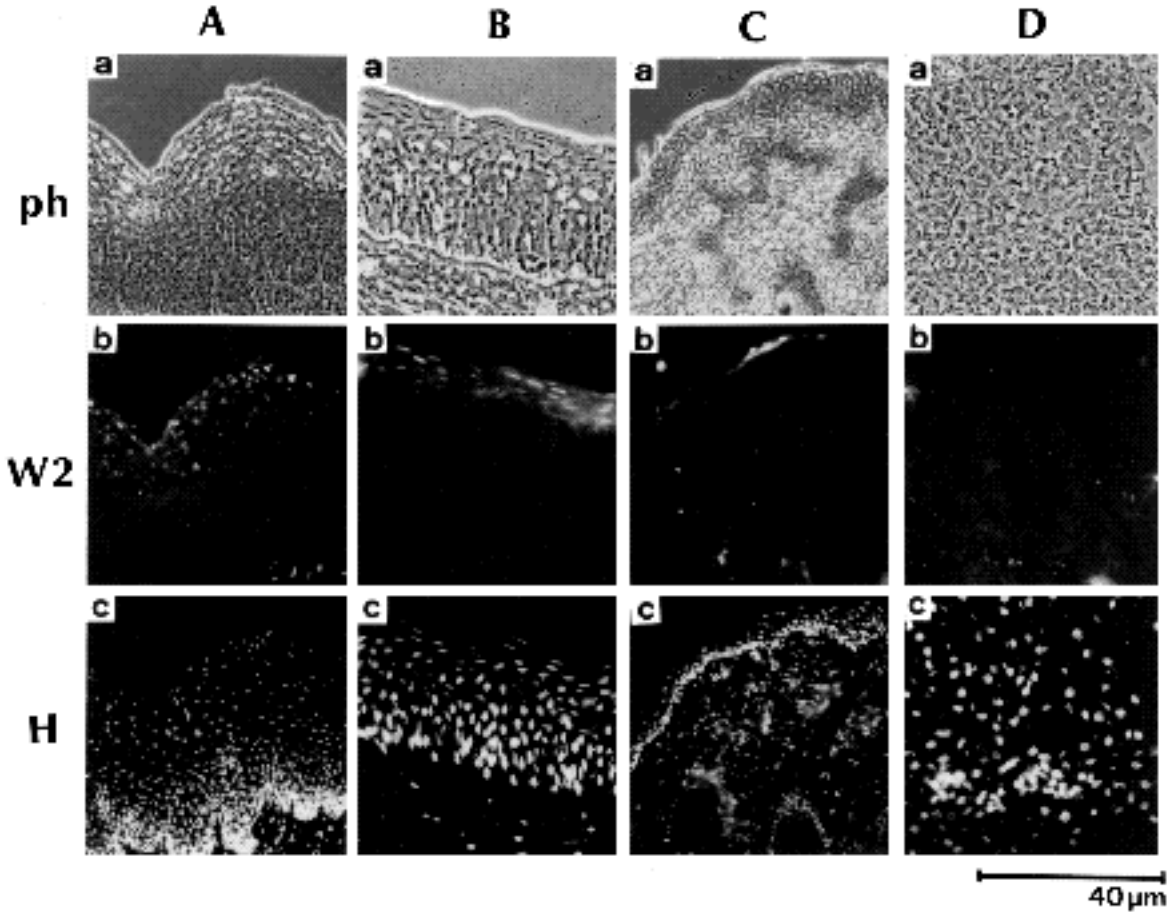

Fig. 2. Reactivity of mAb W2 with cells at various stages of differentiation in bovine tissues: (A) esophagus, (B) cornea, (C) epidermis and (D) liver. The tissue samples were embedded in a "sandwich" for frozen sections, fixed in methanol/acetone (1:1 v/v) at −20ºC, incubated with supernatant of hybridoma W2, followed by fluorescein-conjugated, goat anti-mouse immunoglobulin. Included in the last incubation was the DNA-specific dye, Hoechst 33258. ph, phase contrast micrographs; W2, immunofluorescence of mAb W2; H, staining of DNA by Hoechst 33258. Bar, 40 µm.

differences in staining patterns between different mAbs served as parallel controls during our screening for hybridoma.

Development of the W2 antigen in cultured BEE cells

The in vitro proliferative potentials of small, medium and large cells, isolated after Percoll gradient fractionation, were tested in culture using mitomycin C-treated 3T3 fibroblasts as a feeder layer. Consistent with the notion that small cells were derived from the proliferative basal layer of the in vivo tissue, we found that these cells can proliferate vigorously in vitro. When intially plated at high density, a large number of small colonies were observed. Initial plating at low density, however, gave rise to a small number of large colonies. In contrast to these results obtained with the small cells, the medium and large cells exhibited little growth when cultured under the same conditions.

When BEE tissue was dissociated by Dispase and trypsin digestion and then subjected to in vitro culture, a mixture, consisting predominantly of small cells and some medium cells, was attached to the culture dish, while large cells did not (Fig. 3a). Some of the cells in the colonies developed into large cells, with distinctly condensed nuclei (Fig. 3d). At the level of light microscopy, they showed morphology characteristic of cornification, similar to those observed in frozen sections of the BEE tissue and in the large cells isolated after Percoll gradient fractionation (Fig. 1). The appearance of large cells with condensed nuclei amongst a colony of other cells was noticeable after 2-3 weeks, and was prominent by the fourth week in culture.

Quite strikingly, when the cultured BEE cells were subjected to immunofluorescence staining with mAb W2, there was little or no staining after a short term culture (10 days) (Fig. 3c). However, there was distinct mAb W2 labeling of the condensed nuclei when these cultures were allowed to develop for three weeks (Fig. 3f). Although the fields chosen for the micrographs in Fig. 3 show a difference in the number of cells between 10-day (3a-c) and 21-day (3d-f) cultures, comparisons of the corresponding cultures in the same experiment and in other experiments (stained with other monoclonal antibodies) do not indicate a substantial "dying off" of the cells. The appearance of mAb W2 staining in BEE cells as a function of time in culture should be contrasted with the disappearance of staining due to mAb A1, which specifically recognizes an antigen in the proliferative basal cells (unpublished observation). In this connection, it should also be noted that, while mAb A1 stained several cultured cell lines of diverse origin, including human esophageal carcinoma cell lines TE-9 and CE-48, human glioma cell line U-251 and mouse 3T3 fibroblasts, mAb W2 failed to label any of these cells.

Identification and sequence analysis of the target antigen of mAb W2

To identify the target antigen, a 2 M NaCl extract of large cells of BEE, prepared by the same procedure as the original immunogen fraction, was incubated with mAb W2. Because mAb W2 is a mouse IgG capable of binding to protein A, protein(s) of the large cells of BEE reactive with the mAb was immunoprecipitated. Under non-reducing conditions, SDS-PAGE analysis of the W2-bound material yielded the following upon Coomassie blue staining: (1) a band of M_r ~33,000 and (2) a broad area of staining at the top of the gel, with M_r >100,000, which most probably corresponds to the W2 immunoglobulin molecule (Fig. 4A, lane 1). Electrophoresis of the same sample in the presence of DTT yielded polypeptides of: (1) M_r ~55,000, (2) M_r ~25,000, (3) M_r ~22,000 and (4) M_r ~11,000 (Fig. 4A, lane 2). The bands of M_r ~55,000 and ~25,000 correspond to the heavy and light chains of immunoglobulins, derived from the reduction of the >100,000 M_r band seen in lane 1 of Fig. 4A. This conclusion is supported by the results of a mock immunoprecipitation, where PBS was used in place of cell extract. In this case, only the heavy and light chains of the W2 immunoglobulin molecule were observed upon SDS-PAGE under reducing conditions.

These results indicate that the target antigen of the mAb W2 consists of a protein of M_r ~33,000 under nonreducing conditions and that this protein dissociates into two component polypeptides of M_r ~22,000 and ~11,000 upon reduction, most probably of disulfide linkages. This was

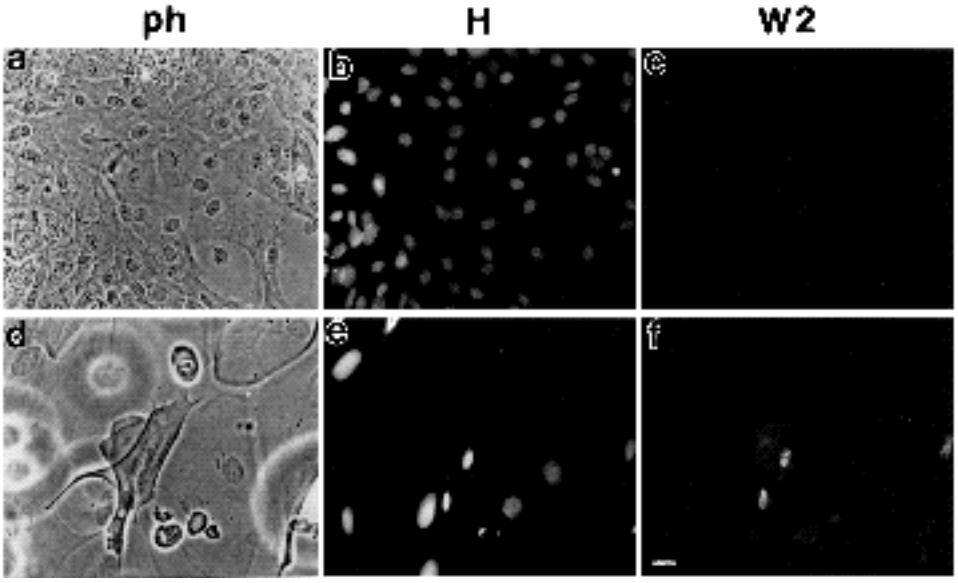

Fig. 3. Reactivity of mAb W2 with primary cultures of BEE. (a,b,c) after 10 days of in vitro culture. (d,e,f) after 21 days of in vitro culture. The cells were fixed with methanol/acetone (1:1 v/v) at −20°C, incubated with the supernatant of hybridoma W2, followed by fluorescein-conjugated, goat anti-mouse immunoglobulin. ph, phase contrast micrograph; H, staining of DNA by Hoechst 33258; W2, immunofluorescence of mAb W2. Bar, 10 μm.

confirmed by the following analysis of the immunoprecipitate derived from mAb W2. After SDS-PAGE under non-reducing conditions, the band of M_r ~33,000 (hereafter designated as BEE33) was eluted from the gel slice corresponding to its position of migration (see Fig. 4A, lane 1). Half of the sample was re-electrophoresed under the same nonreducing conditions; this yielded a single band of M_r ~33,000 (Fig. 4B, lane 1). The other half of the BEE33 sample was re-electrophoresed in the presence of DTT. The results showed that upon reduction, BEE33 yielded polypeptides of M_r ~22,000 (designated BEE22) and M_r ~11,000 (designated BEE11) (Fig. 4B, lane 2).

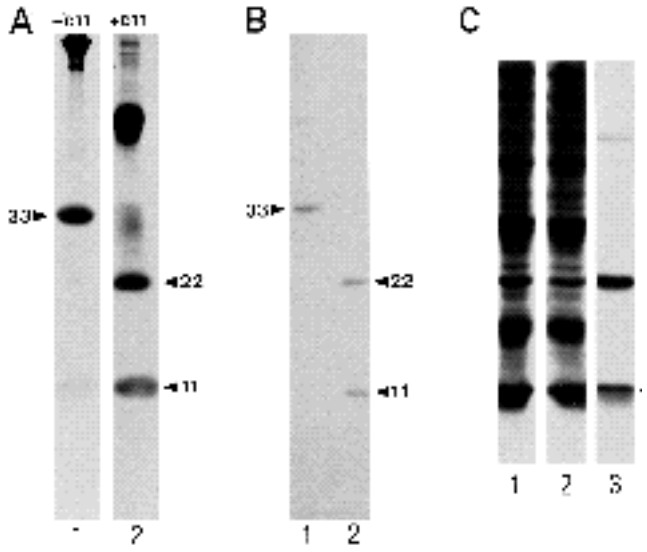

Fig. 4. SDS-PAGE analysis of the polypeptides immunoisolated by mAb W2. (A) Lane 1: immunoprecipitate of mAb W2 electrophoresed under non-reducing conditions. Lane 2: immunoprecipitate of mAb W2 electrophoresed in the presence of DTT. (B) Lane 1: BEE33, eluted from the gel of the mAb W2 immunoprecipitate (A, lane 1), re-electrophoresed under nonreducing conditions. Lane 2: BEE33, eluted from the gel of the mAb W2 immunoprecipitate (A, lane 1), re-electrophoresed in the presence of DTT. (C) Lane 1: a 2 M NaCl extract of large BEE cells was dialyzed against PBS. The supernatant material that was subjected to affinity chromatography was electrophoresed. Lane 2: material not bound to the column. Lane 3: material bound to the column and eluted with glycine-HCl, pH 3.0. Samples were analyzed on 17.5% acrylamide gels and the polypeptides were revealed by Coomassie blue staining. The arrowheads highlight the positions of migration of the bands of M_r ~33,000, ~22,000 and ~11,000 .

The target antigen can be purified by affinity chromatography on an Affi-Gel 10 column covalently derivatized with mAb W2. The immunogen fraction, i.e. the 2 M NaCl extract of large BEE cells, was applied; most of the proteins did not bind to the column (Fig. 4C, lanes 1, 2). The bound fraction consisted of two polypeptides on SDS-PAGE under reducing conditions. The positions of migration correspond to those of BEE22 and BEE11 (Fig. 4C, lane 3).

Amino acid sequence analyses were carried out on BEE22 and BEE11, individually purified from SDS gels. The Edman reaction on intact BEE22 yielded no identifiable amino acid, suggesting that the amino terminus was blocked. A substantial amount of the polypeptide sequence of BEE22 was obtained, however, from peptide fragments derived from trypsin, V-8 and clostripain digests. The sequence of BEE22 was found to be highly homologous to the heavy chain of the leukocyte L1 complex (Fig. 5). The latter is a calcium-binding protein (M_r ~ 36,500) identified in leukocytes, macrophages and epithelial cells (Andersson et al., 1988). It consists of a heavy chain (M_r ~13,000) and a light chain (M_r ~8,300) whose amino acid sequences have been shown to be identical to the subunits of a myeloid-related protein designated MRP14 and MRP8, respectively (Andersson et al., 1988; Lagasse, 1991; Odink et al., 1987). Finally, sequence analysis of the cystic fibrosis (CF) antigen, a serum protein elevated in CF patients, showed almost complete identity to MRP8 (Dorin et al., 1987). For simplicity of nomenclature, we will hereafter refer to this group of small calcium-binding proteins in terms of the heavy and light chains of the L1 complex. The major difference between BEE22 and the heavy chain of the L1 complex is the length of the polypeptides; while the L1 heavy chain contains 114 residues, BEE22 contains at least 122 residues. Over the region of overlap, there was 75% identity between the two proteins (Fig. 5). Like the heavy chain of the L1

```
                1              -α-helix-<--- turn ---->α-helix--   50
BEE 22              M.SQME SSIETIINIF HQYSVRLGHY DTLIQKEFKQ LVQKELPNFL
Human L1-h      mtckM.SQlE rnIETIINtF HQYSVkLGHp DTLnQgEFKe LVrKdLqNFL
Mouse L1-h      mankapSQME rSItTIIdtF HQYSrkeGHp DTLskKEFrQ mVeaqLatFm
Consensus       ------SQ-E --I-TII--F HQYS---GH- DTL---EF-- -V---L--F-

               51       -α-helix-<--- turn -->α-helix--          100
BEE 22          KKQKKNEAAI NEIMEDLDTN VDKQLSFEEF IMLVARLTVA SHEEMHNTAP
Human L1-h      KKenKNEkvI ehIMEDLDTN aDKQLSFEEF IMLmaRLTwA SHEkMHe.gd
Mouse L1-h      KKeKrNEAlI NdIMEDLDTN qDnQLSFEEc mMLmAkLifA cHEklHennP
Consensus       KK---NE--I --IMEDLDTN -D-QLSFEE- -ML-A-L--A -HE--H----

               101                     127
BEE 22          PGQGHRHGPG YGKGGSGSCS GQGSPDQ
Human L1-h      eGpGHhHkPG lGeGtp
Mouse L1-h      rGhGHsHGkG cGK
Consensus       -G-GH-H--G -G-------- -------
```

Fig. 5. Comparison of the amino acid sequence of bovine BEE22 with the heavy chains of the human and mouse L1 complex. The amino terminus of BEE22 was blocked and we failed to isolate the amino-terminal tetrapeptide; therefore, this sequence is missing in the present comparison. The amino acid sequences of human and mouse L1 heavy chain (L1-h) were taken from Lagasse (1991). Amino acid residues identical to those in BEE22 are shown in capital letters; amino acid residues different from the corresponding residues in BEE22 are shown in lower case letters. A period (.) in the sequences of BEE22 and L1-h indicates that a gap was introduced for optimal alignment. The consensus sequence on the last line displays those residues that show complete identity for the three sequences available. The positions of the helix-turn-helix motif are highlighted at the top.

```
                1           α-helix-<--- turn ----->α-helix--        50
BEE 11          MLTDLExAIN ILIDVYHKYS LKKGNYHAV
Human L1-l      MLTeLEkAlN siIDVYHKYS LiKGNfHAVy rddlkkllet ecpqyirkkg
Mouse L1-l      MpseLEkAls nLIDVYHnYS niqGNhHAly kndfkkmvtt ecpqfvqnin
Consensus       M---LE-A-- --IDVYH-YS ---GN-HA-- ---------- ----------

                α-helix-<--- turn --> α-helix-               93
BEE 11
Human L1-l      advwfkeldi ntdgavnfqe flilvikmgv aahkkshees hke
Mouse L1-l      ienlfreldi nsdnainfee flamvikvgv ashkdshke. ...
Consensus       ---------- ---------- ---------- ---------- ---
```

Fig. 6. Comparison of the amino acid sequence of bovine BEE11 with the light chains of the human and mouse L1 complex. The sequence of the first 29 residues of BEE11 was determined by subjecting the intact polypeptide to sequence analysis. The amino acid sequences of human and mouse L1 light chain (L1-l) were taken from Lagasse (1991). The reference sequence used here is that of BEE11. Amino acid residues identical to those in BEE11 are shown in capital letters; amino acid residues different from the corresponding residues in BEE11 are shown in lower case letters. The letter x in the sequence of BEE11 indicates an amino acid could not be identified for that residue. The consensus sequence on the last line displays those residues that show complete identity for the three sequences available. The positions of the helix-turn-helix motif are highlighted at the top.

complex, BEE22 contains two calcium-binding sites, characterized by the well-defined helix-turn-helix motif designated as the EF-hand (Kretsinger, 1980).

Like the L1 protein, the W2 antigen consists of a disulfide-bonded complex of two distinct subunits. It was no surprise, then, to find homology between BEE11 and the light chain of the L1 protein. Amino acid sequence analysis of intact BEE11 showed that 23 out of the first 29 residues were identical to the human L1 light chain (Fig. 6). Overall, there could be little doubt concerning the relationship between the W2 antigen and the L1 complex, as well as its family members. For this reason, we have deferred the completion of the amino acid sequence of BEE11 at the protein level, in favor of isolating the cDNA clones corresponding to the BEE11 and BEE22 polypeptides.

mAb W2 and polyclonal antibodies: comparison by immunoblotting and immunofluorescence analyses

A 2 M NaCl extract of large cells of BEE was dialyzed against PBS and then heated for 5 min at 80°C. The supernatant was subjected to western blotting with mAb W2 under nonreducing conditions. A single band corresponding to BEE33 was revealed (Fig. 7, lane 1). In contrast, parallel SDS-PAGE analysis in the presence of DTT followed by immunoblotting, failed to yield any W2-reactive material (not shown). The western blot of BEE33 was specific, i.e. control blots with an irrelevant mAb M2 or with PBS did not yield the band of M_r ~33,000.

Polyclonal rabbit antisera were generated against BEE22 and BEE11, isolated separately from SDS gels. The BEE33 band detected by mAb W2 under non-reducing SDS-PAGE conditions could also be immunoblotted by rabbit anti-BEE22 or rabbit anti-BEE11. When the W2 antigen isolated from the immunoaffinity column (Fig. 4C) was subjected to SDS-PAGE under reducing conditions and western blotting with rabbit anti-BEE22, only BEE22 was observed (Fig. 7, lane 2). Preimmune rabbit serum analyzed in parallel gave no reaction (not shown). Conversely, rabbit anti-BEE11 stains only BEE11 under reducing conditions (Fig. 7, lane 3). Thus, there does not appear to be any

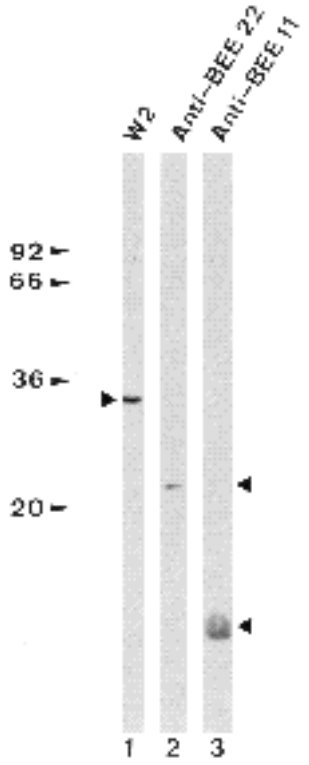

Fig. 7. Immunoblotting analysis with mAb W2 and polyclonal antibodies. Lane 1: a 2 M NaCl extract of large BEE cells was heated to 80°C for 5 min. The supernatant fraction was subjected to SDS-PAGE under non-reducing conditions and western blotting with mAb W2. Lane 2: the W2 antigen isolated from the immunoaffinity column (Fig. 4C) was subjected to SDS-PAGE under reducing conditions and western blotting with rabbit anti-BEE22. Lane 3: the W2 antigen isolated from the immunoaffinity column (Fig. 4C) was subjected to SDS-PAGE under reducing conditions and Western blotting with rabbit anti-BEE11. The material was analyzed on 17.5% acrylamide gels and the polypeptides were revealed as described in Materials and Methods. The numbers on the left indicate the positions of migration of molecular mass markers ($\times 10^{-3}$). The arrowheads highlight the positions of migration of the M_r ~33,000, ~22,000 and ~11,000 bands.

immunological cross-reactivity between BEE22 and BEE11.

When frozen sections of BEE tissue were subjected to immunofluorescence staining with rabbit anti-BEE22, prominent labeling of the superficial layer of large cells and little or no labeling of the basal layer was observed. This pattern was the same as that obtained with mAb W2 (Fig. 2). Moreover, double immunofluorescence, using fluorescein-conjugated, goat anti-mouse immunoglobulin to detect mAb W2 binding and rhodamine-conjugated, goat anti-rabbit immunoglobulin to detect rabbit anti-BEE22 binding simultaneously, showed that individual nuclei labeled with mAb W2 were also labeled with rabbit anti-BEE22 (Fig. 8).

Essentially the same results were obtained when the corresponding analysis was carried out with rabbit anti-BEE11. This antiserum showed staining only in the very superficial layer of BEE. In double immunofluorescence experiments, the staining obtained with rabbit anti-BEE11 was less intense than that of mAb W2 (Fig. 9). As a result, some of the nuclei labeled with mAb W2 showed little or no staining with the polyclonal antiserum. Nevertheless, each nucleus showing rabbit anti-BEE11 staining was also labeled with mAb W2. These colocalization results confirm the identification of the polypeptides of M_r ~22,000 and ~11,000 polypeptides as the components of the antigen recognized by mAb W2 in large cells in the superficial layer of BEE. They argue against the notion that one or both of the polypeptides identified by immunoprecipitation and immunoblotting are artifacts of the 2 M NaCl extraction procedure.

Because immunofluorescence staining patterns may be prone to masking of epitopes, the segregation of the W2 antigen to the large cells of the superficial layer was confirmed by immunoblotting. On the basis of equal amounts (20 μg) of total protein electrophoresed, isolated large cells yielded at least 8 times more BEE22 than fractions enriched in small cells. The Percoll gradient-derived small cell fraction was routinely contaminated by other cell types, including large cells (see Table 1). This could account for the low levels of BEE22 detected in the small cell fraction.

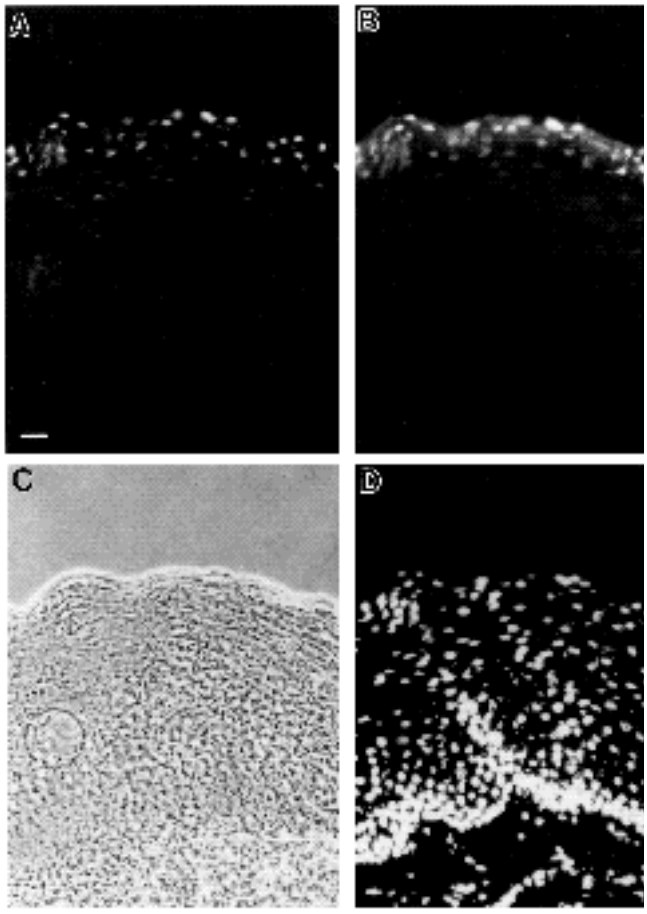

Fig. 8. Double immunofluorescence staining of BEE tissue by mAb W2 and polyclonal rabbit anti-BEE22. Frozen sections of BEE were fixed in methanol/acetone (1:1 v/v) at −20°C, incubated with antibody reagents, followed by fluorescein-conjugated, goat anti-mouse immunoglobulin or rhodamine-conjugated, goat anti-rabbit immunoglobulin. Included in the last incubation was the DNA-specific dye, Hoechst 33258. (A) Fluorescein fluorescence of mAb W2; (B) rhodamine fluorescence of rabbit anti-BEE22; (C) phase contrast micrograph; (D) staining of DNA by Hoechst 33258. Bar, 5 μm.

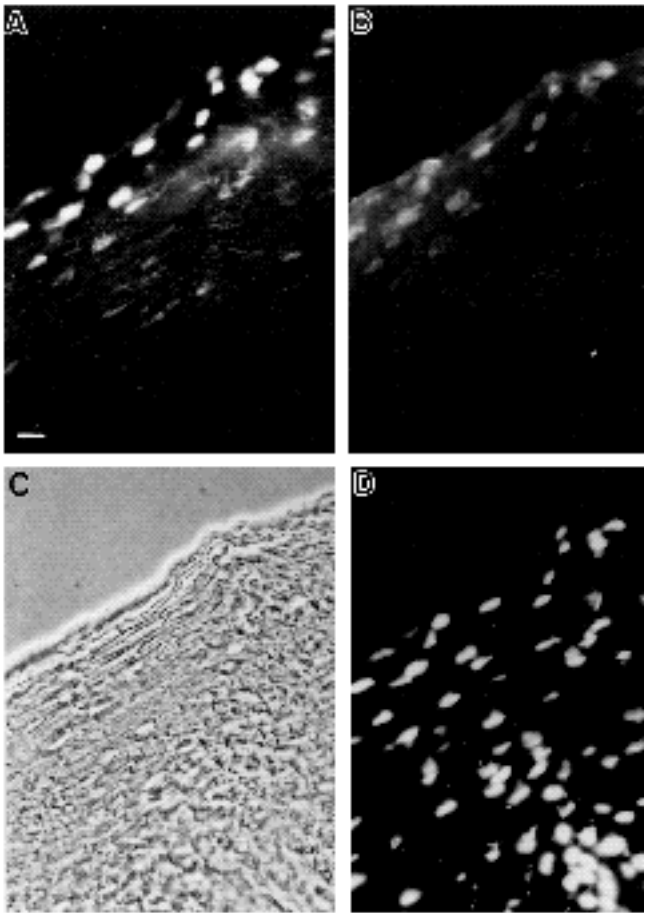

Fig. 9. Double immunofluorescence staining of BEE tissue by mAb W2 and polyclonal rabbit anti-BEE11. The protocol of Fig. 8 was used. (A) Fluorescein fluorescence of mAb W2; (B) rhodamine fluorescence due to rabbit anti-BEE11; (C) phase contrast micrograph; (D) staining of DNA by Hoechst 33258. Bar, 10 μm.

DISCUSSION

Among stratified epithelia, the epidermis, cornea and esophagus exemplify three distinct programs of differentiation. Although the composition, morphology and ultimate differentiated fate of the cells of these tissues differ (Green et al., 1982), the growth features of the cells in the stratified layers of the epithelia do appear to share a common scheme: stem cell → transient amplifying cell → terminally differentiated cell (Lavber and Sun, 1982, 1983). Components of the nucleus and their organization surely play key roles in the pathway of differentiation, as well as in the regulation of the proliferative potential of the cells. Thus, our understanding of the divergence of the differentiation program and the apparent convergence of the proliferative potential of the epithelial cells could be enhanced by identifying proteins of the cell nucleus specific for a given stage of differentiation.

One approach to the identification of such proteins is to generate a bank of hybridoma-secreting antibodies directed against stage-specific, nuclear components. Bovine esophageal epithelium was the system chosen for our study, mainly because of the ready availability of large amounts of material for biochemical characterization. Cells representing various stages of differentiation of esophageal epithelia were fractionated and nuclei or fractions containing nuclear proteins derived from these cells were used as immunogens for hybridoma production. Since the keratin polypeptides represent major protein components of epithelial cells (Eichner et al., 1984), we wished to obviate the problem of generating a large number of hybridoma clones directed against this cytoskeletal structure. Particular care was, therefore, taken to use immunogen fractions relatively free of keratin contamination.

Several distinct groups of monoclonal antibodies have been identified. The first group includes antibodies that stain condensed nuclei of highly differentiated cells in the superficial layer of the epithelium; this group of hybridoma is exemplified by mAb W2 reported in the present paper. A second group includes antibodies specific for nuclear components that are only observed or are present at elevated levels in proliferating cells of the basal layer; this group of hybridoma is exemplified by mAb A1 (unpublished data). Finally, a third group of antibodies appears to be directed at subnuclear structures such as the mitotic spindle, as reported in the accompanying manuscript (Tang et al., 1993).

Hybridoma W2 was generated with an immunogen fraction consisting of a 2 M NaCl extract of the large cells, representing the highly differentiated superficial layer of BEE. The following key features of the target antigen of mAb W2 are documented in the present study. Firstly, the target antigen, immunoprecipitated from the original immunogen fraction, is a protein of M_r ~33,000 (BEE33), consisting of two polypeptides (BEE22, M_r ~22,000 and BEE11, M_r ~11,000) linked by interchain disulfide bonds. The amino acid sequence of BEE22 shares extensive homology to the heavy chain of L1 complex, a calcium-binding protein. The homology is concentrated to the metal ion-binding domain; the conservation of the characteristic helix-turn-helix motif (EF-hand) (Kretsinger, 1980) is particularly striking. On the other hand, the carboxy-terminal end is less conserved, an observation similar to the comparison of mouse and human homologs of the L1 complex (Lagasse, 1991). In the present comparison of bovine BEE22 versus human and mouse L1 protein, it was found that the former has a much longer (~40 amino acids) carboxy-terminal tail, based on the deduced amino acid sequence of BEE22 cDNA clone (unpublished data). The partial sequence of BEE11 also shows striking homology/identity to the light chain of L1 protein. Preliminary evidence indicates that BEE33, as well as each of the component polypeptides (BEE22 and BEE11), can bind $^{45}Ca^{2+}$ (unpublished data), as assayed by the method of Charuk et al. (1990). Thus, the subunit structure of BEE33, the sequence of the BEE22 and BEE11 component polypeptides and their binding properties all show strong analogy to the L1 complex.

Both the W2 antigen and the L1 complex, in turn, belong to the S-100-like calcium-binding protein family, including S-100a, S-100b, Calcyclin (2A9), 42A, 42C and p9Ka (Lagasse and Clerc, 1988). The expression of some of the S-100-like proteins is associated with cell differentiation. Mammary cuboidal stem cells are p9Ka-negative, but become p9Ka-positive after differentiation into myoepithelial-like cells (Baraclough et al., 1987). Nerve growth factor treatment of PC12 cells induces the synthesis of 42A and 42C, after the appearance of a neural phenotype (Masiakowski and Shooter, 1988). Finally, the L1 complex (MRP14 and MRP8) expression is associated with myelopoiesis (Lagasse, 1991). Thus, the identification of the W2 antigen in the differentiated superficial cells of esophageal and corneal epithelia adds to the list of systems of cell differentiation in association with the expression of S-100-like proteins.

Secondly, mAb W2 recognized BEE33 in western blots under non-reducing conditions, but failed to recognize either BEE22 or BEE11 in reducing gels. Thus, the epitope for mAb W2 could be a structural determinant formed by interaction between BEE22 and BEE11. Alternatively, mAb W2 could recognize a conformational epitope on one of the polypeptides constrained by disulfide bonds. It should be noted that mAb W2, as well as polyclonal rabbit anti-BEE22 and rabbit anti-BEE11, colocalize in the condensed nuclei of large cells of BEE tissue. This argues against the possibility that one of the polypeptides actually comes from a different part of the cell, but is coprecipitated as an artifact of the 2 M NaCl extract.

Thirdly, the target antigen of mAb W2 appears to be associated with the condensed nuclei of terminally differentiated cells. This is manifest not only through examination of the large cells of BEE derived from the in vivo tissue, but also from inducing the conversion of BEE small cells into large cells in culture. mAb W2 also stained the condensed nuclei of the in vitro differentiated large cells. The apppearance of mAb W2 labeling in BEE cells as a function of time in culture should be contrasted with the disappearance of staining due to mAb A1, which labels a nuclear antigen in proliferative basal cells. It should be noted, however, that the absence of staining by mAb A1 in a given cell does not necessarily lead to staining with mAb W2. This conclusion is supported by analysis of the level

of staining of mAb A1 in cultured cell lines, whose proliferative state can be manipulated by serum growth factors. For example, quiescent 3T3 fibroblasts show little or no mAb A1 staining. Subsequent serum stimulation of the same cells yielded nuclear labeling by the mAb A1. mAb W2, on the other hand, failed to label any of these cells. Moreover, the data available thus far suggest that the target antigen of mAb W2 is restricted to epithelial cell types; no staining of cells of mesenchymal origin, such as fibroblasts in the connective tissue layer of BEE, has been observed.

The target antigen of mAb W2 is not restricted to esophageal epithelium. It is also found in the highly differentiated, superficial layer of corneal epithelium. There was little staining due to mAb W2 in the superficial layer of the epidermis, most probably because the final stages in that differentiation program occur quickly, with rapid elimination of the nucleus. In this connection, it would be of interest to test whether mAb W2 can recognize the condensed nuclei of avian erythrocytes, in which nuclear condensation has taken place, but nuclear elimination has not. Analysis of proteins specifically associated with the condensed nucleus and characterization of their endogenous function/activity may shed light on the process of nuclear condensation and elimination.

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