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A simple epithelial cell line (MDCK) shows heterogeneity of desmoglein isoforms, one resembling pemphigus vulgaris antigen

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SUMMARY

The epidermal blistering disease, pemphigus vulgaris (PV), is caused by circulating autoantibodies that react with a desmosomal glycoprotein desmoglein (Dsg3). This antigen is expressed only in stratified epithelial tissues. Here we show that the simple epithelial canine kidney cell line, MDCK, expresses at least two desmoglein isoforms recognised by different monoclonal antibodies. One of these isoforms is a $130 \times 10^3 M_r$ polypeptide that is recognised by both PV autoantisera and a monoclonal antibody reactive

with a cytoplasmic domain of human Dsg3. Antibodies in PV sera bind to the surface of MDCK cells but do not cause loss of intercellular adhesion. This is the first demonstration of the expression of a polypeptide related to human PV antigen by a simple epithelial cell type.

Key words: desmosome, cell adhesion, desmoglein, pemphigus vulgaris, MDCK cell

INTRODUCTION

The desmogleins are desmosomal glycoproteins that constitute a subfamily of the cadherin superfamily of calcium-dependent adhesion molecules (Buxton and Magee, 1992; Magee and Buxton, 1991; Legan et al., 1992; Garrod, 1993). There are three desmoglein isoforms known from human tissues, referred to as Dsg1, -2 and -3 (Buxton et al., 1993). Characteristically they possess a cadherin-like extracellular domain, a single trans-membrane region and a large cytoplasmic domain containing a series of unique 29 amino acid repeats. In Dsg1 and 2, but not Dsg3, the extracellular domain is about 50 amino acids shorter in the membrane proximal region than that of a conventional cadherin. The size of the cytoplasmic domain is variable, containing 5 repeats in Dsg1, 6 in Dsg2, but only 2 in Dsg3 (Wheeler et al., 1991; Koch et al., 1991; Amagai et al., 1991).

Dsg3 was originally characterised as the pemphigus vulgaris antigen (PVA), the target antigen of autoantibodies that cause the epidermal blistering disease, pemphigus vulgaris (Eyre and Stanley, 1988; Stanley, 1993). Pemphigus blistering, caused by loss of desmosomal adhesion between epidermal keratinocytes, is normally restricted to the epidermis and oral mucosa, and does not affect other epithelial tissues. IgG autoantibodies from the sera of pemphigus patients cause loss of adhesion (acantholysis) between keratinocytes in culture and epidermal blistering when injected into new born mice (Farb et al., 1978; Woo et al., 1983; Diaz et al., 1986). These antibodies show wide cross-reactivity with the epidermis of mammalian species, but have not been found to react with simple epithelia (Diaz et al., 1978).

We have previously described a monoclonal antibody, 32-2B, raised by immunising mice with desmoglein (probably a mixture of isoforms) purified from bovine nasal epidermis and specially selected for reactivity with formaldehyde-fixed, paraffin-embedded, pathological specimens (Vilela et al., 1987). This antibody proved extremely reliable as a diagnostic marker for carcinomas (Vilela et al., 1987; Conn et al., 1990; Harada et al., 1992), but was unsuitable for use in immunofluorescence of tissue culture cells. This led us to develop another monoclonal antibody to desmoglein, 33-3D (characterised for the first time here), suitable for immunofluorescence studies. Subsequent immunoblotting studies with these two monoclonal antibodies showed that they are heterogeneous in their polypeptide reactivity with various cells and tissues.

We show here that 32-2B recognises PVA as well as higher molecular mass desmoglein(s) in human epidermis, and, surprisingly, a polypeptide that resembles PVA in the simple epithelial canine kidney cell line MDCK, which expresses at least two desmoglein isoforms. Although mRNA for Dsg3 has been found (Schäfer et al., 1994), this is the first time that expression of a PVA-like glycoprotein has been demonstrated in simple epithelial cells.

MATERIALS AND METHODS

Cell lines

Madin-Darby bovine and canine kidney cells (MDBK and MDCK) were cultured as described previously (Cowin et al., 1984; Matthey and

Garrod, 1986). The KU8 penile carcinoma cell line and its culture have been described previously (Tsukamoto, 1989).

Monoclonal antibodies

The production and characterisation of 32-2B has been described in detail (Vilela et al., 1987). 33-3D was obtained from the same fusion as 32-2B. The hybridomas were cloned six times by limiting dilution. 32-2B belongs to subclass Ig2a while 33-3D was typed as IgM. Average Ig concentrations of tissue culture supernatants were found by ELISA to be 100 µg/ml for 32-2B and 70 µg/ml for 33-3D. Monoclonal antibody HECD-1 to human E-cadherin was kindly provided by Professor M. Takeichi.

Gel electrophoresis and western blotting

Polyacrylamide gel electrophoresis (PAGE) and western blotting were carried out using bovine epidermal desmosomal cores (Gorbsky and Steinberg, 1991) or cytoskeletal extracts of tissue culture cells (Fey et al., 1984) as described previously (Suhrbier and Garrod, 1986). Nitrocellulose bearing extracts of human keratinocytes was kindly supplied by Dr J. D. Aplin (St Mary's Hospital, Manchester, UK). Preparation of human epidermal extracts and immunoblotting of these was described by Hashimoto et al. (1990). Non-equilibrium pH gradient electrophoresis (NEPHGE) was carried out according to O'Farrell (1975) and O'Farrell et al. (1977). 2-D gels were blotted as described for 1-D gels by Suhrbier and Garrod (1986).

Pemphigus autoantisera

These were obtained from patients at the Department of Dermatology, Keio University, Tokyo, Japan, and were characterised by immunofluorescence and immunoblotting. Control sera were obtained from normal subjects. Five different PV sera and three control sera were used.

Fluorescent antibody staining

Procedures for staining cultured cells with monoclonal antibody were as described previously (Mattey et al., 1990). For staining with pemphigus autoantisera cells were fixed in methanol at -20°C for 20 minutes and then incubated sequentially in serum diluted 1:40 and FITC anti-human IgG at 1:100 for 1 hour at room temperature before washing in PBS and mounting.

To test the ability of autoantisera to disrupt cell adhesion, cells were incubated at 37°C in sera diluted 1:10 with tissue culture medium for 2 hours. They were then washed in PBS and fixed in 3% formaldehyde in PBS, washed and stained with FITC anti-human IgG at 1:100 for 1 hour at room temperature before washing and mounting.

Immunoelectron microscopy

Small (0.5-1 mm³) pieces of bovine nasal epidermis were fixed in freshly made 2% formaldehyde in 200 mM Hepes buffer, pH 7.4, for 1 hour at room temperature. The tissue was then infused for 2 hours with a polyvinylpyrrolidone/sucrose mixture (Tokuyasu, 1989) prior to freezing in liquid nitrogen. Ultrathin sections (80-100 nm) were cut using a Reichert Ultracut E microtome with FC4D cryoattachment, retrieved on droplets of 2 M sucrose plus 0.5% gelatin in Hepes buffer and transferred to Formvar-coated 100 mesh nickel grids.

Tissue sections were blocked with 2% gelatin in PBS, then treated with 0.02 M glycine in PBS to quench free aldehyde groups, and further blocked with 5% normal goat serum plus 1% BSA in PBS. The primary antibodies, monoclonals 33-3D and 32-2B, were applied as neat supernatant for 1 hour at room temperature. After thorough washing, the grids were then incubated on 5 nm colloidal gold conjugated to goat anti-mouse IgM or goat anti-mouse IgG antibodies, respectively (Biocell Research Laboratories, Cardiff) for 30 minutes at room temperature. After further washes, the sections were post-fixed in 2.5% glutaraldehyde for 5 minutes, and washed with water (4 times, 1 minute). They were subsequently stained for 5 minutes in

2% uranyl acetate/oxalate, then briefly rinsed in water prior to staining and embedding in aqueous 0.2% uranyl acetate plus 2% polyvinylalcohol (Tokuyasu, 1989). Microscopy was performed using a Philips 400 or JEOL 1200 transmission electron microscope, operated at 80 or 100 kV.

Cell trypsinisation

Trypsinisation of living cells for immunoblotting was carried out as described by Parrish et al. (1990).

RESULTS

Monoclonal antibodies to desmogleins

Antibody 32-2B has been described previously (Vilela et al., 1987). Its reactivity and that of the new antibody, 33-3D, with desmosomal core polypeptides are shown in Fig. 1. Both antibodies show specific reactivity with the desmoglein band (probably a mixture of isoforms). In order to confirm that both monoclonal antibodies react with the same desmoglein polypeptide blots were carried out on desmosomal cores separated in two dimensions by NEPHGE (Fig. 2A). The pattern of desmosomal polypeptides seen by this procedure is similar to that previously reported (Mueller and Franke, 1983). Note that both 32-2B (Fig. 2A, lane A) and 33-3D (Fig. 2A, lane B) react with the same spot. Confirmation that both antibodies reacted with the same major spot was obtained by

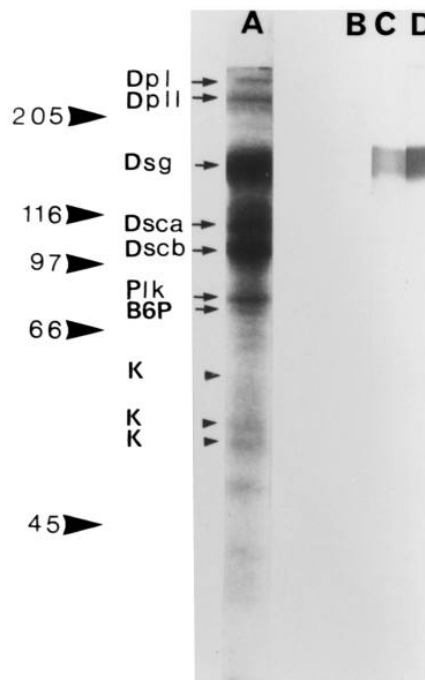


Fig. 1. Reactivity of monoclonal antibodies with desmosomal core polypeptides. Lane A, Coomassie Blue staining of desmosomal core polypeptides (DpI and II, desmoplakins; Dsg, desmogleins; Dsca and b, desmocollins; Plk, plakoglobin; B6P, band 6 protein K, keratin). The gel is heavily overloaded to demonstrate the specificity of the antibodies. Lanes B-D, autoradiographs of nitrocellulose strips corresponding to A. B, control, no first antibody; C, 32-2B; D, 33-3D. M_r values ($\times 10^{-3}$) are given on the left.

reacting both antibodies with the same blot (Fig. 2B, lanes A,B,C,D). No additional spots were revealed by this procedure. It is concluded that both 32-2B and 33-3D are specific for desmoglein of bovine nasal epidermis.

Both monoclonal antibodies gave characteristic punctate peripheral fluorescent staining of keratinocytes in frozen sections of bovine nasal epidermis (not shown). However, neither antibody reacted with living or formaldehyde-fixed MDCK cells. When the cells were permeabilized by fixation with acetone, 33-3D gave punctate peripheral staining characteristic of desmosome reactivity (Fig. 3A). 32-2B, on the other hand, gave only very weak peripheral staining, which was difficult to discern clearly (Fig. 3B). These results suggest that 33-3D and probably 32-2B react with cytoplasmic epitopes.

32-2B and 33-3D react with different-sized products in MDCK and other cells

Immunoblotting of cytoskeletal extracts of MDCK cells with monoclonal antibodies 32-2B and 33-3D revealed different patterns of polypeptide reactivity (Fig. 4). 32-2B reacted with a single band of $130 \times 10^3 M_r$ (Fig. 4, lane B) while 33-3D showed reactivity with a band (probably a doublet) of $150 \times 10^3 M_r$ and also with a $130 \times 10^3 M_r$ band apparently similar to that recognised by 32-2B (Fig. 4, lane C). These results suggest that MDCK cells possess a complement of heterogeneous desmoglein polypeptides.

Immunoblotting experiments were also carried out with MDBK cells, human keratinocytes and HN5 cells from a squamous cell carcinoma of head and neck (Cowley et al., 1986). 32-2B does not react with MDBK cells but 33-3D

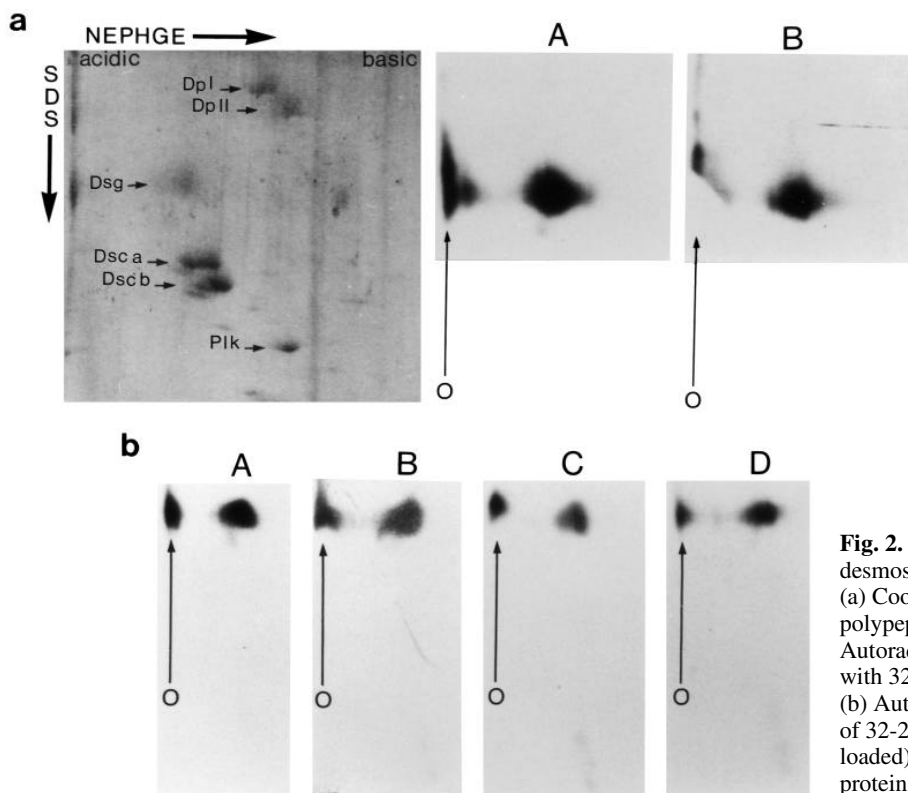


Fig. 2. Reactivity of monoclonal antibodies with desmosomal core polypeptides on 2-D gels. (a) Coomassie Blue staining of desmosomal core polypeptides (labelling as Fig. 1). (A and B) Autoradiographs of nitrocellulose corresponding to gel with 32-2B (A) and 33-3D (B). O, origin. (b) Autoradiographs of nitrocellulose showing reactivity of 32-2B (A) and 33-3D (B) together (50 µg protein loaded); (C and D) 32-2B and 33-3D together (25 µg protein loaded).

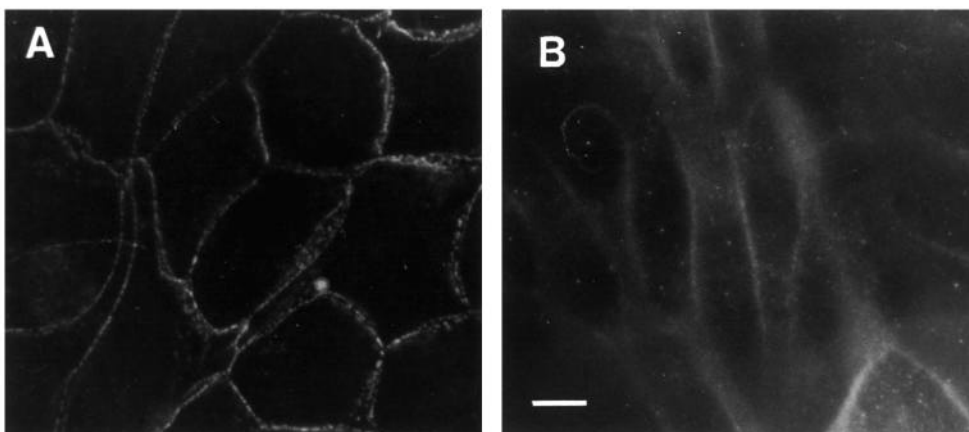


Fig. 3. Fluorescent staining of acetone-fixed MDCK cells with monoclonal antibodies 33-3D (A) and 32-2B (B). Bar, 20 µm.

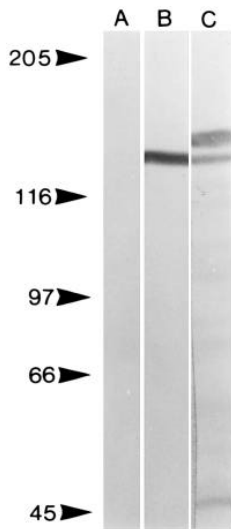


Fig. 4. Immunoblots showing reactivity of monoclonal antibodies with MDCK cells. (A) Control, no first antibody; (B) 32-2B; (C) 33-3D. $M_r (\times 10^{-3})$ on the left.

recognises a single band of mobility intermediate between those recognised by the same antibody in MDCK cells (Fig. 5A, lanes B and C). In keratinocytes (Fig. 5B) and HN5 cells (not shown) 32-2B recognises bands of 150 kDa and 130 kDa, while 33-3D recognises only the upper one of these bands.

In order to confirm the reactivity of 32-2B with PVA a series of immunoblotting experiments have been carried out. Firstly, 32-2B has been used to immunoblot bacterial fusion proteins corresponding to the cytoplasmic domains of Dsg1 and Dsg3 and shown to react with both (Dmochowski et al., 1994; Hashimoto et al., 1995). Secondly, we wished to confirm that 32-2B does not react with E-cadherin, a glycoprotein of similar molecular mass to Dsg3 and present in MDCK cells. Fig. 8 (below) shows immunoblots of human penile carcinoma cell line KU8 with PV patient antiserum, 32-2B and monoclonal antibody HECD-1 to human E-cadherin. Both PV serum and 32-2B recognise a single band of identical mobility whilst HECD-1 recognises a distinct band of slightly greater mobility, which is not recognised by 32-2B. Also 32-2B does not show a band of 150 kDa in KU8 cells, indicating the absence of Dsg1. Confirmation that 32-2B does not recognise E-cadherin was obtained by conducting immunoblots on extracts of L929 cells expressing recombinant E-cadherin. No reactivity with E-cadherin was found. We conclude that 32-2B recognises the cytoplasmic domains of both Dsg1 and Dsg3, and does not react with E-cadherin. Since it does not react with MDCK cells in which Dsg2 is the only desmoglein isoform (Schäfer et al., 1994), we conclude that it does not react with Dsg2.

MDCK desmoglein isoforms show cytoplasmic size heterogeneity

In order to investigate the transmembrane distribution of the polypeptides, whole-cell trypsinisation experiments were carried out in the absence of Ca^{2+} according to the method of Parrish et al. (1990), followed by immunoblotting with the monoclonal antibodies.

The results of blotting trypsinised MDCK cells with 32-2B are shown in Fig. 6A and with 33-3D in Fig. 6B. The $130 \times 10^3 M_r$ polypeptide recognised by 32-2B is reduced by trypsinisation to a membrane protected fragment of about $50 \times 10^3 M_r$. By contrast, the $150 \times 10^3 M_r$ 33-3D polypeptide is digested in two

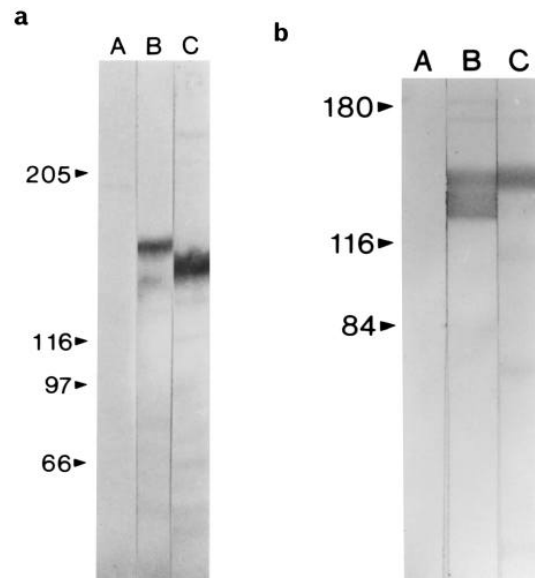


Fig. 5. Immunoblots showing reactivity of monoclonal antibodies with (a) MDCK cells and (b) human keratinocytes. (a) Lane A, 32-2B on MDCK cells; B, 33-3D on MDCK cells; C, 32-2B on MDCK cells. Note 32-2B does not react with MDCK cells. (b) Lane A, control, no first antibody; B, 32-2B; C, 33-3D.

stages, first to a fragment of about $100 \times 10^3 M_r$ and finally to a membrane-protected fragment of about $65 \times 10^3 M_r$. Cells were $>90\%$ viable as shown by trypan blue exclusion following trypsinisation for 90 minutes. Thus the major glycoproteins recognised by these two antibodies show different fragmentation patterns on trypsinisation and yield membrane-protected fragments differing by about $15 \times 10^3 M_r$.

Confirmation that 32-2B and 33-3D react with the cytoplasmic region of desmosomes was obtained by electron microscopy using immunogold labelling of ultrathin cryosections of bovine nasal epidermis (Fig. 7).

PV sera react with MDCK cells

The $130 \times 10^3 M_r$ of the polypeptide recognised by 32-2B in MDCK cells, together with the ability of 32-2B to recognise PVA in epidermal cells, are consistent with the possibility that it is related to PVA. Moreover, cDNA cloning and sequencing of epidermal PVA shows that its cytoplasmic domain is substantially smaller than that of the other desmogleins (Amagai et al., 1991). The MDCK polypeptide also has a smaller cytoplasmic domain, further suggesting its possible relatedness to PVA. In order to investigate this possibility we tested the reactivity of PV sera with MDCK cells by immunofluorescent staining and immunoblotting.

Fluorescent staining of methanol-fixed MDCK cells with PV sera revealed a strong peripheral pattern of reactivity, consistent with a junctional location of the reactive antigen. The distribution seemed generally linear with a few areas showing a punctate pattern possibly consistent with desmosomal location (Fig. 9). This pattern is consistent with the type of staining routinely found with such sera on human epidermis.

In order to determine (a) whether PV sera reacted with the extracellular domains of an MDCK antigen and (b) whether sera would cause loss of intercellular adhesion between MDCK cells, the living cells were incubated with a high concentration

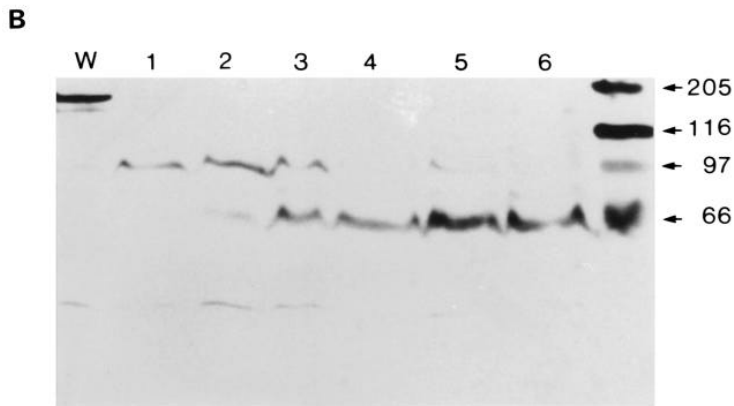
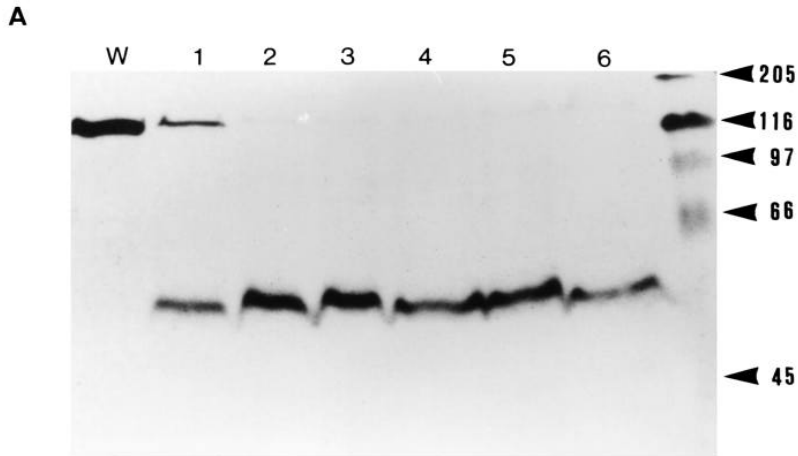


Fig. 6. Immunoblots showing reactivity of monoclonal antibodies on MDCK cells treated with trypsin. (A) 32-2B; (B) 33-3D. Note that the sizes of the trypsin membrane-protected fragments recognised by the two antibodies differ. W, whole, untreated cells; trypsin treatments for 5 minutes (lane 1), 10 minutes (lane 2), 15 minutes (lane 3), 30 minutes (lane 4), 45 minutes (lane 5), 80 minutes (lane 6).

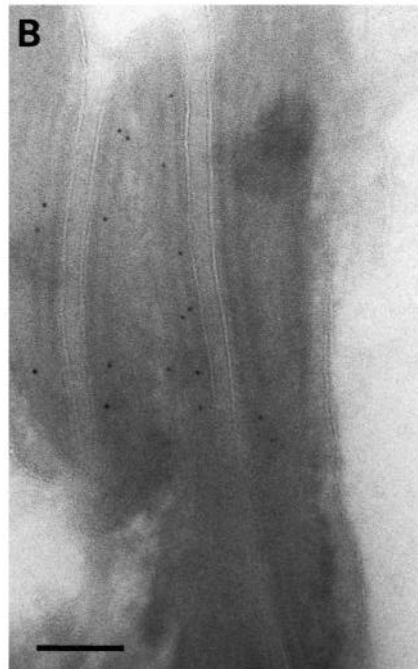
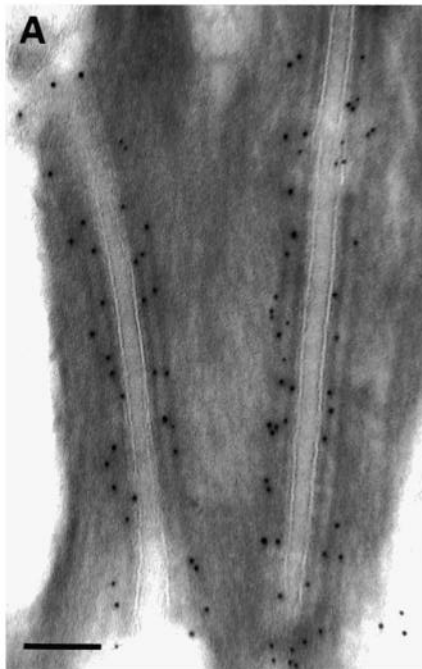


Fig. 7. Immuno-gold labelling of ultra-thin frozen sections of bovine nasal epidermis with (A) 33-3D and (B) 32-2B, showing reactivity of these antibodies with the cytoplasmic plaques of desmosomes. Bar, 100 μ m.

(1:10) of serum at 37°C for 2 hours. The cells were then fixed in formaldehyde and reacted with a FITC-labelled second antibody. The cells showed a strongly peripheral pattern of

staining very similar to that obtained after methanol fixation (Fig. 9). However, there was no indication of loss of intercellular adhesion. The results indicate that PV sera react with an

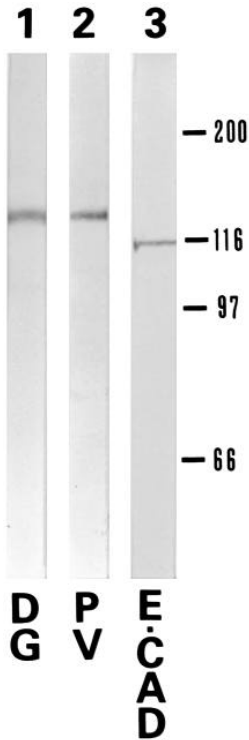


Fig. 8. Monoclonal antibody 32-2B and PV sera do not react with E-cadherin. Extracts of KU8 penile carcinoma cells were immunoblotted with monoclonal antibody 32-2B (lane 1, DG), PV serum (lane 2, PV) and monoclonal antibody HECD-1 to human E-cadherin (lane 3, E-CAD). The bands in lanes 1 and 2 have identical mobilities and are quite distinct from the E-cadherin band in lane 3. Many PV sera have been blotted against KU8 cells and none has been found to react with E-cadherin. M_r ($\times 10^{-3}$) on the right.

antigen on the outer membrane surface of MDCK cells but do not cause loss of intercellular adhesion.

Immunoblotting experiments were carried out on MDCK cells to determine the polypeptide reactivity of PV sera. The results show that all PV sera recognise a band of $130 \times 10^3 M_r$ (Fig. 10, lanes 2-6) that is not recognised by control sera (Fig. 10, lanes 7-9). This polypeptide band has identical mobility to that recognised by monoclonal antibody 32-2B (Fig. 10, lane 1).

DISCUSSION

Our results indicate that the MDCK cell line exhibits heterogeneity of desmoglein isoforms expressing at least two polypeptides of 150×10^3 and $130 \times 10^3 M_r$. The larger polypeptide, because of its size, its large membrane-associated cytoplasmic domain, and its reactivity with a desmoglein-specific monoclonal antibody, possesses characteristics of a desmoglein, resembling human Dsg1 and 2 and bovine Dsg1 (Koch et al., 1990, 1991; Wheeler et al., 1991). Because this larger MDCK polypeptide is not recognised by 32-2B, which reacts with Dsg1 and Dsg3, we conclude that it is most likely Dsg2. The smaller polypeptide appears to be more closely related to human Dsg3, the pemphigus vulgaris antigen, by several criteria: its size, the smaller size of its

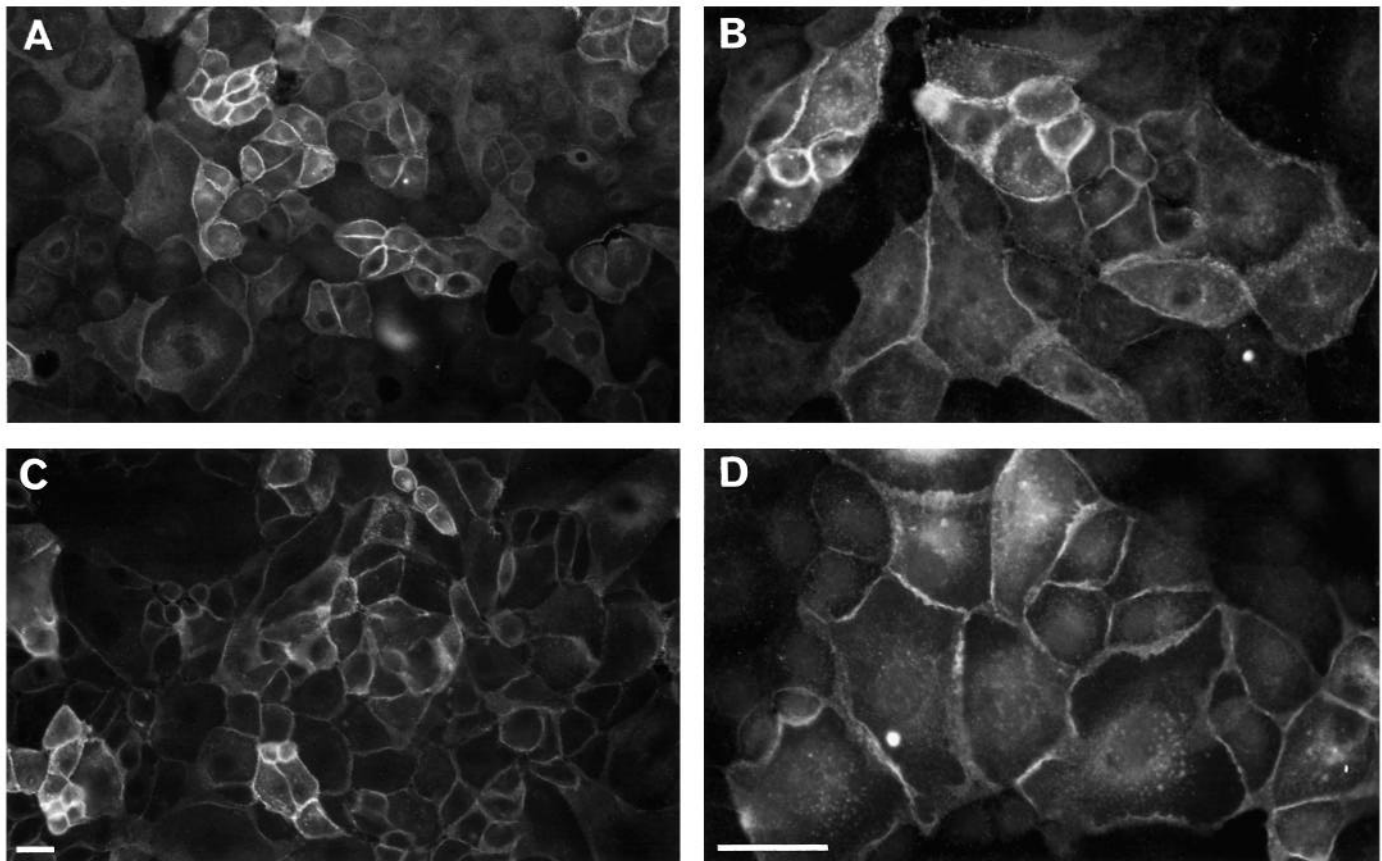


Fig. 9. Fluorescent antibody staining of MDCK cells with pemphigus vulgaris auto-antisera. (A and B) Cells fixed with methanol before staining. (C and D) Cells treated with serum before fixation, then fixed with formaldehyde before staining. Note that in each case the staining is principally concentrated at regions of intercellular contact and there is no apparent difference in antigen distribution or in degree of intercellular contact between the two preparations. There is some indication of punctate staining where the cell boundaries are viewed obliquely (arrowheads). Control human sera gave no staining (not shown). Bars, 20 μm .

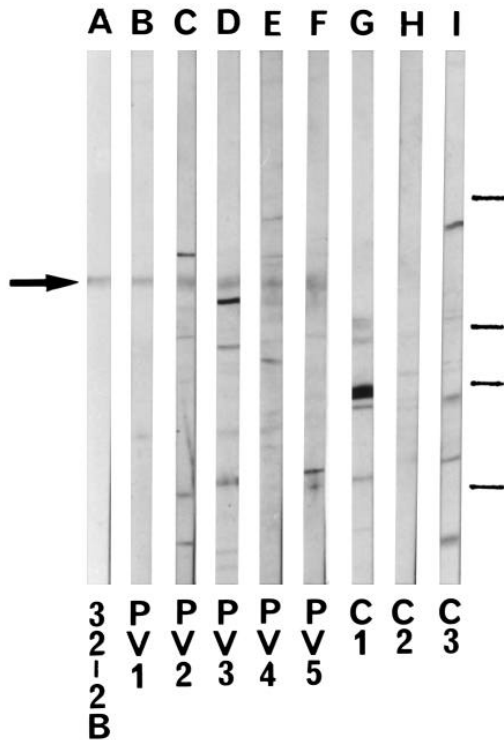


Fig. 10. Immuno-blotting of MDCK cells with pemphigus vulgaris auto-antisera. An extract of MDCK cells blotted with 32-2B (lane A), five different PV sera (lanes B-F), and three control sera (lanes G-I). Note that the five PV sera, although showing spurious reactivity with various other polypeptides, all react with a band of $130 \times 10^3 M_r$ on the left of identical mobility to that recognised by 32-2B. None of the control sera reacts with this band. Relative molecular mass markers as in Fig. 9.

membrane-associated cytoplasmic domain, its reactivity with a monoclonal antibody that recognises PVA, and its reactivity with PV antisera. Schäfer et al. (1994) have recently reported that bovine mammary gland epithelial cells (BMGE) and thymic epithelium express the mRNA of Dsg3. Our work constitutes the first report of the expression of a protein molecule related to PVA in a simple (rather than stratified) epithelial cell type.

Heterogeneity among desmogleins between different cell types and tissues was indicated some time ago by protein studies (Suhbier and Garrod, 1986; Schmelz et al., 1986) and has now been clearly demonstrated by cloning and sequencing of cDNAs. The expression of more than one desmoglein isoform mRNA in certain tissues and BMGE cells has recently been reported (Schäfer et al., 1994). However, this is the first time that Dsg protein heterogeneity has been indicated within a cell line.

Recent studies on the expression patterns of desmosomal glycoproteins, both desmogleins and desmocollins, in stratified epithelia, indicate that cells in tissues also contain mixtures of desmosomal glycoprotein isoforms. Thus, *in situ* hybridisation studies of expression patterns of three desmoglein isoforms in human epidermis (Arnemann et al., 1991) and three desmocollin isoforms in several stratified bovine tissues (Legan et al., 1994) show that the expression territories of different isoform-specific mRNAs show localised regions of maximal expression but nevertheless clearly overlap. Staining epithelia with

isoform-specific monoclonal antibodies to two bovine desmocollins, Dsc1 and Dsc3, clearly indicate that the distributions of the glycoproteins also overlap (Legan et al., 1994; Yue et al., unpublished). Thus the distribution of Dsc1 in epidermis is exclusively suprabasal while that of Dsc3 is strongly, but not exclusively, basal. The latter diminishes gradually for several layers above the basal layer. Since, where the glycoproteins are expressed, antibody staining indicates that they occur in every cell, it is clear that cells in regions where expression overlaps must contain both isoforms. We do not yet have a specific antibody to the third desmocollin isoform, Dsc2, but *in situ* hybridisation suggests that its expression territory overlaps those of the other two, so many cells probably possess the different isoforms (Legan et al., 1994).

These results raise many questions. When different desmosomal glycoprotein isoforms occur in the same cell, do they occur in different desmosomes or are they mixed in the same desmosomes? If they are in separate desmosomes, which desmoglein isoforms occur with which desmocollins, or do they all occur together in the same organelle? What would be the function of the alternative patterns? Presumably, different desmosomes containing distinct sets of desmosomal glycoproteins could have distinctly different adhesive properties. However, the distributions of different isoforms within stratified epithelia suggest that the adhesive properties of the cells at different levels within the epithelium may also change gradually. Thus, there may be desmosomal adhesive gradients spanning the depth of a stratified epithelium. Unfortunately, the very weak staining of MDCK cells with 32-2B and the apparently slight cross-reactivity of 33-3D with the $130 \times 10^3 M_r$ polypeptide do not permit us to determine by fluorescence, confocal or immunoelectron microscopy whether the two polypeptides occur in the same or different desmosomes in these cells, or even whether the PVA-like polypeptide has a desmosomal location.

The occurrence of a PVA-like molecule in a simple epithelial cell is remarkable. Since MDCK are canine cells, it is possible that such an occurrence is unique to dog tissues, or even dog kidney. In order to check this, frozen sections of a range of canine tissues were reacted with PV sera. Reactivity was found only in epidermis, and was clearly absent from kidney. It may be that such expression is a culture artifact, since MDCK cells are highly adapted to culture conditions. However, it may also be that epidermal PVA is one of a family of molecules, and those that occur in other tissues may not be recognised by PV autoantibodies. Since PV serum recognised the $130 \times 10^3 M_r$ in MDCK cells but did not cause acantholysis, it may be that the simple epithelial antigen is subtly different from the epidermal antigen. Cloning and sequencing of the MDCK molecule should resolve this issue.

32-2B monoclonal antibody was shown to recognise a wide range of normal and malignant human epithelial tissues, including many simple epithelia (Vilela et al., 1987; Garrod and Fleming, 1990). It is rather remarkable therefore that it does not recognise the desmoglein of MDBK cells, since the antibody was raised against bovine proteins. It also does not recognise the other desmoglein of MDCK cells, which may indicate further complexity in the desmoglein subfamily. Further characterisation of the epitopes recognised by both antibodies would be valuable.

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