Monoclonal Antibodies Provide Specific Intramolecular Markers for the Study of Epithelial Tonofilament Organization

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ABSTRACT The tonofilament-associated protein antigens recognized in epithelial cells by a group of six monoclonal antibodies have been studied by immunofluorescence and gel immunoautoradiography. The monoclonal antibodies were generated against detergent insoluble cytoskeleton extracts from a cultured simple epithelium derived cell line, PtK_1 cells. They show various tissue specificities, and while they all recognize components at the low end of the molecular weight range for intermediate filament proteins, they confirm that single antibody species can react with multiple polypeptides of different molecular weights in the tonofilament complex. The monoclonal antibodies described here demonstrate the presence of a simple epithelium antigenic determinant associated with intermediate filaments that is not detectable in the specialized cells of squamous and keratinizing epithelia but can reappear in such cells after transformation.

Tonofilaments are the most complex and highly developed of the various classes of intermediate filaments and possibly the most intriguing. As a major cytoplasmic component of all epithelial cells, they appear in the electron microscope as filaments of indeterminate length, aggregated laterally into anastomosing bundles and anchored at the cell periphery by looping into specialized intercellular junctions called desmosomes. This morphology has led to the supposition that tonofilaments may play a central role in maintaining the tensile strength and integrity of epithelial cell sheets.

Tonofilaments have variously been referred to as alpha-keratin filaments (1), keratin (2, 3), prekeratin (4, 5), and cytokeratins (6, 7), in reference to their abundance in epidermal keratinocytes and thus their inevitable involvement in fully differentiated keratin. Among the five classes of intermediate filaments that are now distinguishable biochemically (see Lazarides' review [8]), tonofilaments constitute the most heterogeneous group, the number and size of their multiple polypeptide components varying with the species and tissue of origin (9, 10, 11). In spite of this variability published accounts of antisera recognizing epithelial tonofilaments indicate widespread cross-reactivity (3, 12).

The superimposed differences and similarities between tonofilament proteins have complicated attempts to study their intracellular regulation and function. The data indicate that the heterogeneity is not due to the presence of multiple precursors or to degradation products but to the fact that the keratin fibrous proteins, in epidermis and elsewhere, are the products of large families of genes (13, 14). It may be that, in order to define the individual components of the system satisfactorily, points of reference should be sought within the molecules themselves, as antibody-recognized determinants, because molecular shape should be a more biologically significant parameter than molecular size. Thus the apparent cross-reactivity of epithelial antisera should be examined in more detail.

The technique devised by Köhler and Milstein (15) of fusing immune spleen lymphocytes with a myeloma cell line now allows us to obtain pure monoclonal antibody populations in tissue culture. The use of monoclonal antibodies increases the resolution of immunocytochemistry by permitting the analysis of one antigenic determinant at a time. Such reagents have the additional advantage over conventional antisera of being ho-

A collection of monoclonal antibodies has therefore been raised against tonofilaments of an epithelial cell line in tissue culture, PtK_1 cells. Using these single immunoglobulin species it has been possible to demonstrate unequivocally species-restricted as well as species-shared antigenic determinants on

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tonofilaments, and to show that some of these determinants are absent from normal adult keratinizing epidermis. These reagents therefore serve as markers for the differentiation state of epithelial cells. It is also shown that within a given cell type tonofilament polypeptides of different molecular weights can share the same antigenic determinant, and therefore have some degree of molecular structure in common, which is probably a reflection of the relatedness of their coding sequences.

MATERIALS AND METHODS

Immunization of Mice

These experiments were based on a cultured epithelial cell line, PtK_1 cells, as a reproducible source of antigen both for immunization and for subsequent screening. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Cytoskeleton preparations were made by lysing the cells in a nonionic detergent (1% Nonidet P-40 in phosphate-buffered saline [PBS] at pH 7.5). The insoluble material containing the intermediate filaments was briefly washed and spun in PBS; the pellet was dispersed by sonication and injected intraperitoneally with equal volumes of Freund's complete adjuvant into Balb/c mice, at a dosage of 0.1 ml of a 1 ml suspension of material from 5×10^6 cells per injection. Mice received two or more such injections at 1- to 2-wk intervals, with a final intravenous boost of 0.2-ml suspension without adjuvant administered 72 h before the fusion.

Cell Hybridization

Cell hybridization procedures were essentially those of Kennett et al. (16), ignoring erythrocytes and using Sp2/0-Ag14 nonproducer myeloma cells (17). After fusion, using 30% polyethylene glycol 1,000 (J. T. Baker Chemical Co., Phillipsburg, NJ) plus centrifugation in the absence of serum at pH 7.3, the cells were diluted to 40-ml vol with DMEM plus 15% FCS, the serum (Flow Laboratories [Rockville, MD] lot no. 29101101) selected to support myeloma growth at low cell densities. The cells were plated out over 5–6 × 24-well Linbro tissue culture plates and left for 24 h before the addition of HAT medium to allow for some Sp2 conditioning of the culture medium. Media frequently had to be changed after 48 h due to high initial metabolism of Sp2 cells, but in any case were changed at least twice before screening to ensure removal of lymphocyte produced antibodies. Growth was usually visible in 4–6 d, screening was started at 7–9 d, and in many cases cells were transferred to agarose for cloning by 10 d after fusion.

Screening and Cloning

Cultures were screened for interesting antibody production by indirect immunofluorescence on fixed culture dishes of PtK_1 cells using a miniature assay system which allows for rapid screening of large numbers of samples. Microliter aliquots of test culture supernatants were spotted directly onto plastic tissue culture plates of confluent cells, after the plates had been fixed for 5 min in methanol/acetone (1:1) and air-dried. All incubations and washes were as for standard immunofluorescence (below). The plate of cells was flooded with the second antibody, thus incorporating the second antibody control as a background between the test spots of the first antibodies, (Fig. 1). This method is described in more detail elsewhere (18); it is fast and flexible and requires minimal substrate preparation.

Cloning was done by plating selected hybrid cultures at various densities in 1% Seaplaque (LGT) agarose (Marine Colloids, Rockland, ME), made up in DMEM plus 15% FCS with up to 25% Sp2-conditioned medium as necessary. Feeder cells were not found to be essential. The agarose was allowed to set at room temperature (10 min) before returning the cells to the 37°C incubator. Under these conditions, this agarose induced growth of clones as tight, laterally compressed spheroids of cells, with no peripheral loose cells to contaminate nearby colonies. Clones could be picked by eye with a high degree of confidence after 10–14 d. Colonies were transferred to microwells and could generally be rescreened and recloned in another fortnight. Twice-cloned cells were frozen in 95% FCS plus 5% DMSO as soon as possible; cloning was repeated after thawing.

Antibody Characterization

Although ascites fluid could easily be produced by injecting Pristane-primed mice with hybridoma cells, this source of antibody was treated with caution because intermediate filament antigens are notoriously autoimmunogenic (19, 20) and nonmonoclonal antibodies were readily detectable by double immunodiffusion in all the hybridoma ascites fluids tested. Hybridoma culture supernatants were stored with 0.05% sodium azide at 4°C for a year or more. Antibody was separated from the FCS proteins in the culture supernatant by adsorption on a Sepharose 4B rabbit anti-mouse gamma globulin low-affinity antibody column; pure monoclonal antibody (mAb) from a known subsaturating volume of culture fluid was eluted from the column by citrate buffer (pH 3) and its OD^{280} was measured to calculate the antibody yield of some cell lines. Immunoglobulin typing was done by double immunodiffusion in agarose against antisera specific for heavy chains of IgA, IgM (Litton Bionetics, Inc., Kensington, MD), or IgG (Cappel Laboratories, Cochranville, PA), or for individual mouse IgG subclasses (21).

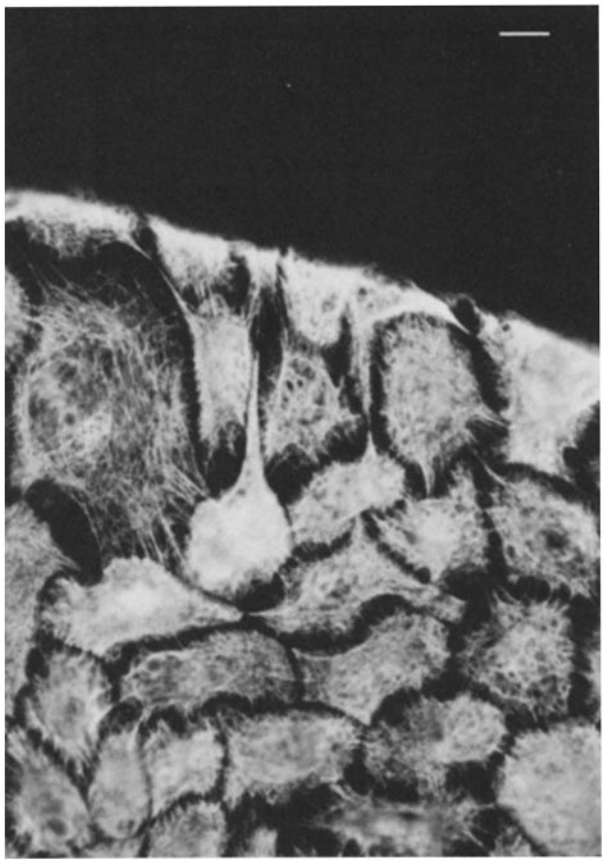

FIGURE 1 Edge of a positive sample from the immunofluorescence screening assay: confluent PtK_1 cells were stained on the plastic culture dish with 1/8 diluted LE65 culture supernatant. In confluent cultures the filaments are very dense and appear as bridges from cell to cell where desmosomes are situated. Outside the drop of the first antibody, at the top, the continuous sheet of cells is unstained by the 2nd layer. Bar, 10 μm.

Immunofluorescence

Cells in tissue culture were grown on cover slips and fixed as for the plate assay, using equal volumes of methanol and acetone. The washing was all done with PBS; mAbs were used as neat or up to 1/8 dilutions of tissue culture supernatants, incubated on the cells for 20 min at room temperature. The second antibody was fluorescein-conjugated goat anti-mouse IgG (heavy and light chain; Cappel Laboratories) filtered × 0.22 μm and diluted 1/40 and incubated for 20 min as above. After washing, the preparations were mounted with Gelvatol 20–30 (Monsanto Petrochemicals, Dayton, OH). Frozen sections 5-μm thick were used either fresh or after washing with 1% NP-40 and similarly stained and mounted. Preparations were examined with a Zeiss Photomicroscope III using UV epillumination and photographed with Kodak Tri-X film uprated to 1,600 ASA. Control samples were examined in parallel with each specimen and assay and consisted of fibroblasts stained identically, or of the same epithelial cell preparations treated with a different mAb, which recognized a nonfilamentous component (often a nuclear antigen) and thus gave a distinctly different staining pattern.

Immunoautoradiography

The cytoskeleton preparations used for immunizing the mice were also used as the sample material in the one-dimensional (22) and two-dimensional (23) polyacrylamide gel electrophoresis. The 2-D gels were kindly run by S. Blose and D. Meltzer (Cold Spring Harbor Laboratory). Fixed and washed gels were stained overnight with antibody following the method described by Burridge (24), using tissue culture supernatants from twice-cloned hybridoma cells. Distribution of the antibody binding sites was marked by a second layer of ^{125}I-rabbit anti-mouse gamma globulin using 5×10^6 cpm/ml for 6 h. Gels were washed for 72 h after each antibody layer in 0.05 M Tris-HCl, pH 7.5 + 0.15 M NaCl + 0.1% sodium azide. After staining with Coomassie Blue they were dried and exposed for 1–4 d at −70°C to Kodak XR-1 x-ray film with an intensification screen. Life-size photographic prints of the autoradiographs were carefully traced over a light box and the tracings accurately superimposed on same size prints of the relevant Coomassie Blue-stained gels to identify the main sites of antibody reactivity. These areas were outlined on top of the Coomassie prints, and all the prints were then trimmed and mounted (Fig. 6).

RESULTS

Selection of Antibody-producing Hybrid Cells

The chosen immunogen, which was a sonicated suspension of the detergent-insoluble material from PtK_1 cells, proved to be very effective in eliciting a good antibody response in Balb/c mice in preparation for the spleen cell-myeloma hybridizations. The fusions characteristically gave one or more clones of viable hybrids or "hybridoma" cells in around 100/300 wells with ~50–60% of cultures producing antibodies to tonofilament-associated components. No attempt was made to purify individual antigen components from the cytoskeleton preparation because this was unnecessary: the purification of the mixture of antibodies produced was done at a later stage, when the hybridoma cells were cloned. By using an immunofluorescence assay system adapted to handle large sample numbers (18), one could quickly distinguish between antibodies to filaments and antibodies to other cellular components at the first screening step. The basketwork-like tonofilament pattern is clearly recognizable in immunofluorescence of cultured epithelial cells by the appearance of intercellular "bridges" at the sites of desmosomes (see Figs. 1, 3) and by the relative resistance of these filaments (25) to Colcemid-induced aggregation (not shown) to which the other intermediate filament types are susceptible. Six vigorous hybridoma cell lines were selected, which secreted antibodies giving the tonofilament staining pattern in indirect immunofluorescence on PtK_1 cells, and were cloned at least twice in soft agarose. No heterogeneity was seen at any third cloning. Cloning was repeated after thawing frozen cells to maintain a high antibody production level; all the lines, however, appear to be quite stable.

The immunoglobulin type of each monoclonal antibody was determined (Table I) by double immunodiffusion against immunoglobulin class and subclass specific antisera. All six monoclonal antibodies are IgG; LE64 and LE65 are IgG2a, whereas LE61, LE62, LE63, and LE41 are IgG1. Each of the six cloned hybridoma tissue culture supernatants gave a single, sharp band with one, and only one, of the subclass-specific antisera, whereas ascites fluids and whole sera gave multiple precipitin lines. The characteristics of their secreted monoclonal antibodies are summarised in Table I.

Reaction with Cells in Culture

All the antibodies react with PtK_2 cells which, like PtK_1 cells, are derived from kidney epithelium of the marsupial *Potorous tridactylis* (26). Although all six antibodies gave a similar tonofilament pattern of antigen localization in PtK_1 cells, there were minor cytoplasmic distribution differences which could be discerned between some of them at low mag-

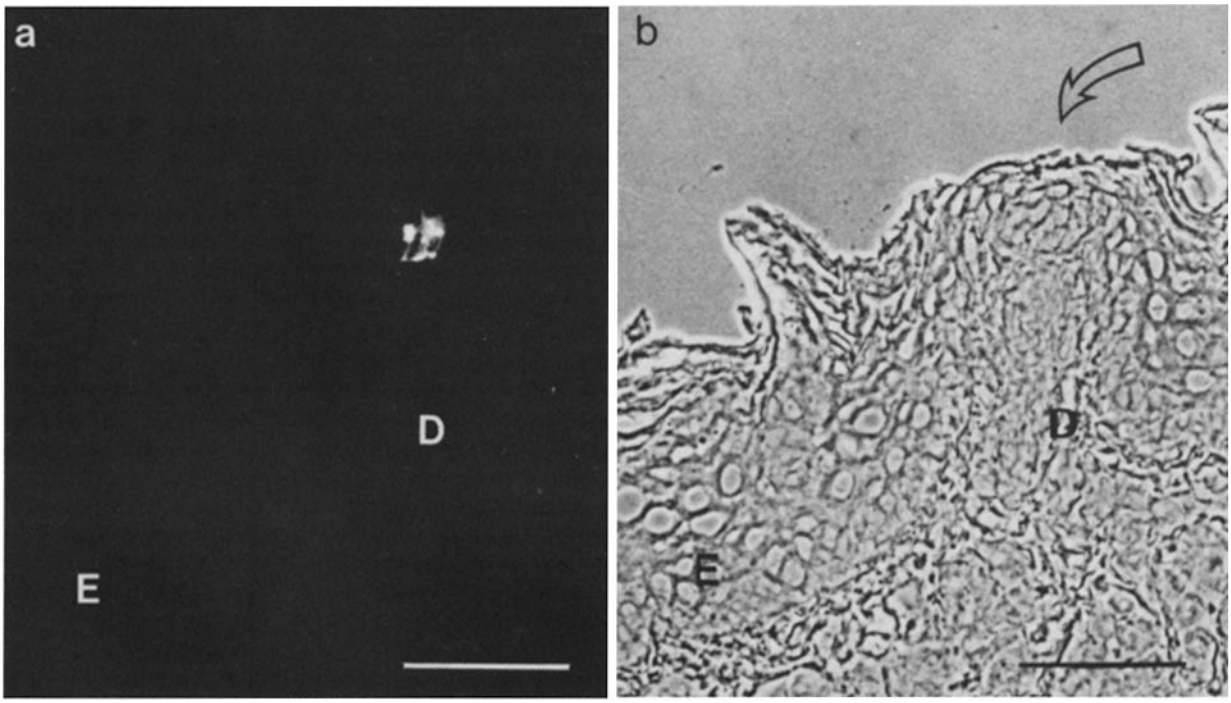

FIGURE 2 Frozen section of adult rat tongue, dorsal surface, through epidermis containing a taste bud sectioned obliquely (arrow) on top of a dermal papilla (*D*) through which nerves and blood supply reach the sensory organ. Cells in the taste bud are stained with LE65 (similarily LE61, not shown), whereas the epidermal keratinocytes (*E*) are not. The same area is shown by immunofluorescence (*a*) and phase contrast (*b*). Bar, 50 μm.

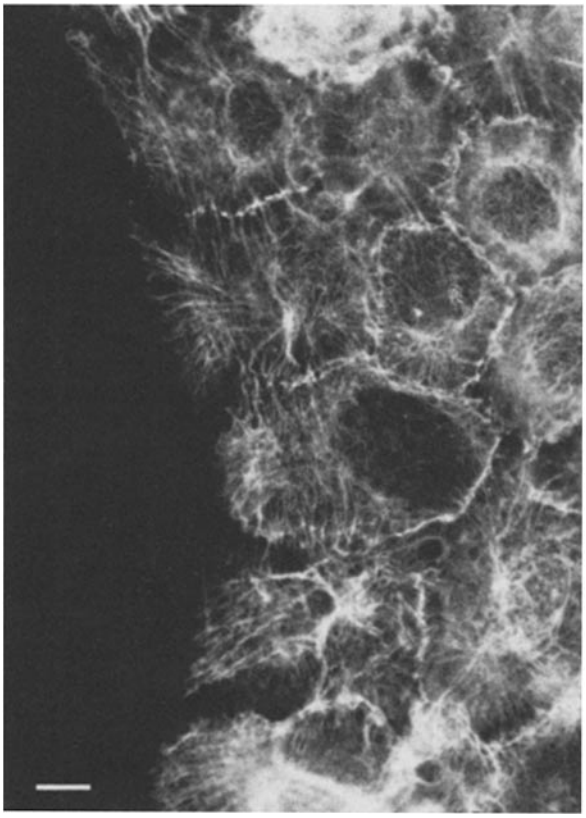

FIGURE 3 Tonofilaments stained in SV40-transformed human keratinocytes with mAb LE61: desmosomes are quite numerous. LE65 gives an identical pattern in this case. Bar, 10 μm.

nifications, particularly between LE64 and LE65. Although it has not been possible so far to qualify these differences by double immunofluorescence or by competition assays, they are confirmed by clear differences in the gel staining patterns and in the cross-reactivity characteristics of the different monoclonal antibodies, (see below).

When other epithelial cell cultures were tested, restricted cross-reactivity was seen. Primary cultures of mouse kidney epithelium were stained by LE61, LE65, and also LE41. BS-C-1 and CV-1 cells are also from kidney epithelium, from the African green monkey: three mAbs (LE61, LE65, and LE41) reacted strongly with these and two (LE63 and LE62) only very weakly. LE64 appears to react only with PtK cells. On HeLa cells (human cervical carcinoma, squamous epithelium), only LE61 and LE65 were positive. Differentiating human keratinocyte cultures among fibroblast feeder cells (courtesy of I. McKay, Imperial Cancer Research Fund, London, England) were not stained by any of the mAbs, whereas an SV40-transformed human keratinocyte cell line (27) was intensely stained by both LE61 and LE65, (Fig. 3).

All nonepithelial cells tested were negative with all six mAbs; these included 3T3 (mouse fibroblasts), IMR 133 (gerbil fibroma cells), PC12 (rat pheochromocytoma line), and L6 (rat myoblasts).

Reaction with Frozen Sections

None of the mAbs reacted with any nonepithelial tissue in fixed or unfixed frozen sections of mouse and rat material. The results with epithelial cells divide the antibodies into three groups: three out of the six (LE64, LE62, and LE63) have not been found to react with any frozen sections, one (LE41) reacts with kidney collecting tubules, and the other two, LE61 and LE65, are widely cross-reactive with other epithelial cells (Table I).

Both LE61 and LE65 reacted strongly with most soft, or simple, non-squamous internal epithelia (e.g. trachea, bronchioles, and alveoli of lung, bile ducts of liver, mammary gland, thymus epithelium, bladder) and moderately with others (intestinal epithelia). In contrast these antigenic determinants were not detectable in keratinocytes or in *stratum corneum* of the epidermis under any of the preparative conditions used, i.e. untreated fresh tissue, methanol/acetone fixed or extracted with 1% NP-40. This result was obtained with all keratinizing and squamous epithelia tested, from human as well as rat and mouse epidermis, buccal epithelium, and esophagus. The basal layer where the epidermal stem cells are was also negative, although an occasional LE61-positive single cell was observed, particularly in the frozen sections of neonatal mouse snout epidermis. In the adult rat and mouse tongue the cells of the taste buds, which are non-keratinizing epidermal cells, were brightly stained by LE61 and LE65, in contrast to the unstained surrounding epidermis (Fig. 2).

Immunoautoradiography of Gels

To identify the antigenic target of each mAb the immunizing material was separated into its constituent polypeptides by SDS-PAGE and the gels stained directly (24) with mAbs,

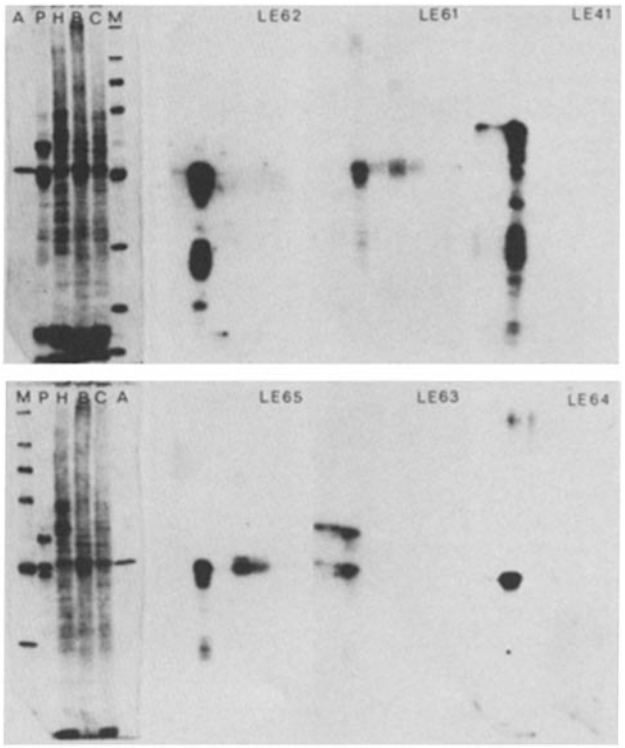

FIGURE 4 Immunoautoradiographs of 10% polyacrylamide gels of detergent-insoluble cytoskeleton extracts to show the reaction of each of the six mAbs. The Coomassie Blue-stained gels shown on the left side correspond directly to the adjacent autoradiographs; the other two (right) are from similar tracks of the same slab gels respectively. *A*, chicken gizzard actin; *M*, molecular weight markers: 200, 180, 94, 68, 45, 30, 21 and 14.3 mol wt ($\times 10^3$); *P*, PtK_1 (potaroo kidney); *H*, HeLa (human cervical carcinoma); *B*, BS-C-1; and *C*, CV-1 (monkey kidney) cells.

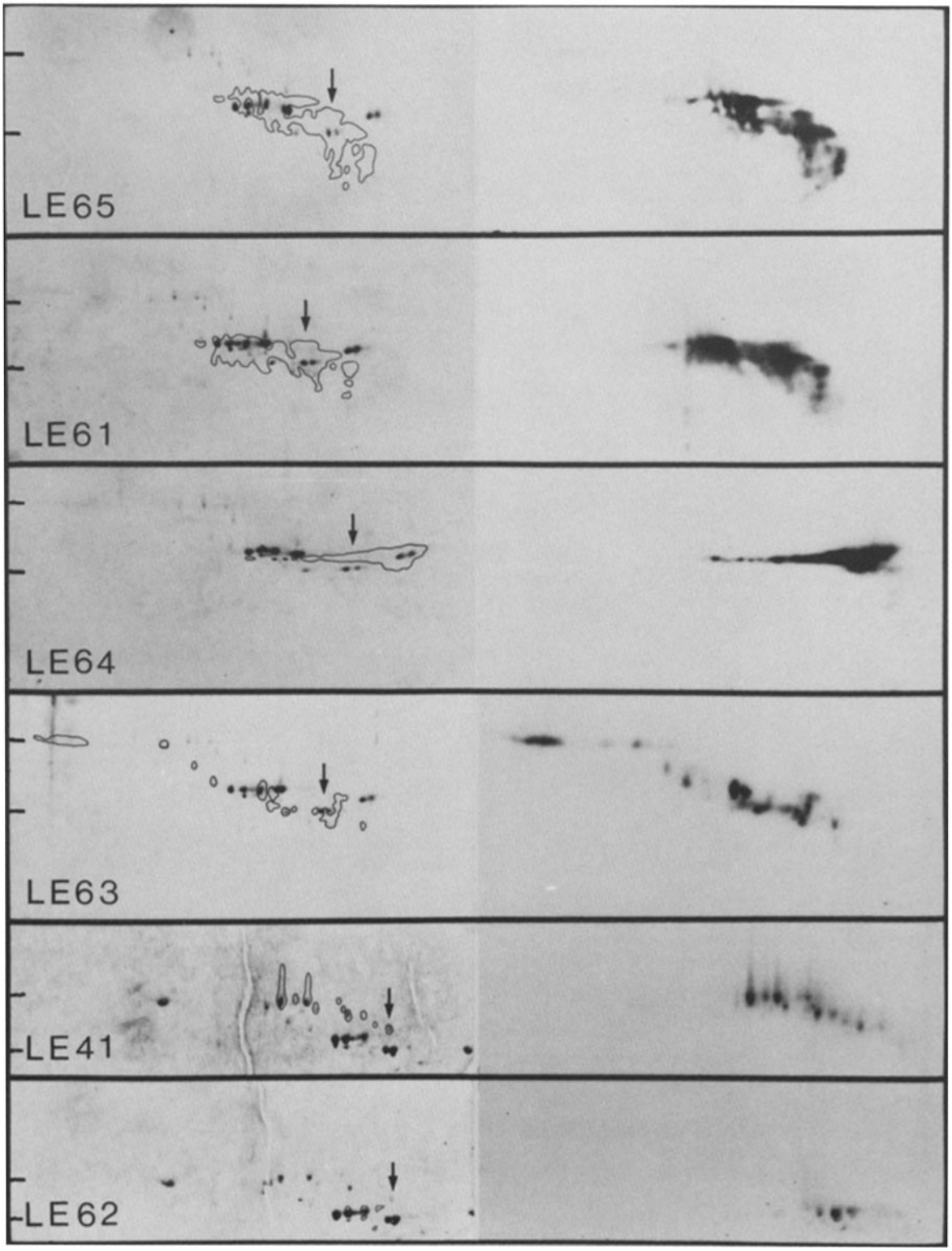

FIGURE 5 Immunoautoradiographs of the six mAbs binding to 2-D gels of PtK_1 cell insoluble extracts as used for Figs. 4 and 6. Coomassie Blue-stained gels (*left*) are marked to show the exact position of the major mAb binding sites. Autoradiographs (*right*) were produced by staining with mAb-containing cell supernatants followed by ^{125}I-labeled rabbit anti-mouse Ig. Markers on left-hand edge indicate extent of migration in the vertical (molecular weight) dimension of polypeptides of 52,000 (upper) and 41,000 mol wt (lower). The bottom two gels have been displaced down and to the right, so that the "D" spots (stained by LE64) are at the edge of the figure. Arrow points towards the major pair of spots marked A_1 and A_2 in Fig. 6.

whose binding was monitored by autoradiography of a radioactive 2nd antibody (Fig. 4). In the Coomassie Blue-stained gels the crude cytoskeleton extract of PtK_1 cells gave 4–5 major bands between 41 and 52,000 mol wt, a 52,000 mol wt band often appearing as a doublet and 3 bands between 41 and 44,000 mol wt (Figs. 4, 6). This is in the lower part of the mol wt range reported by others for keratin-associated fibrous proteins, but cultured epithelial cells do appear to express only

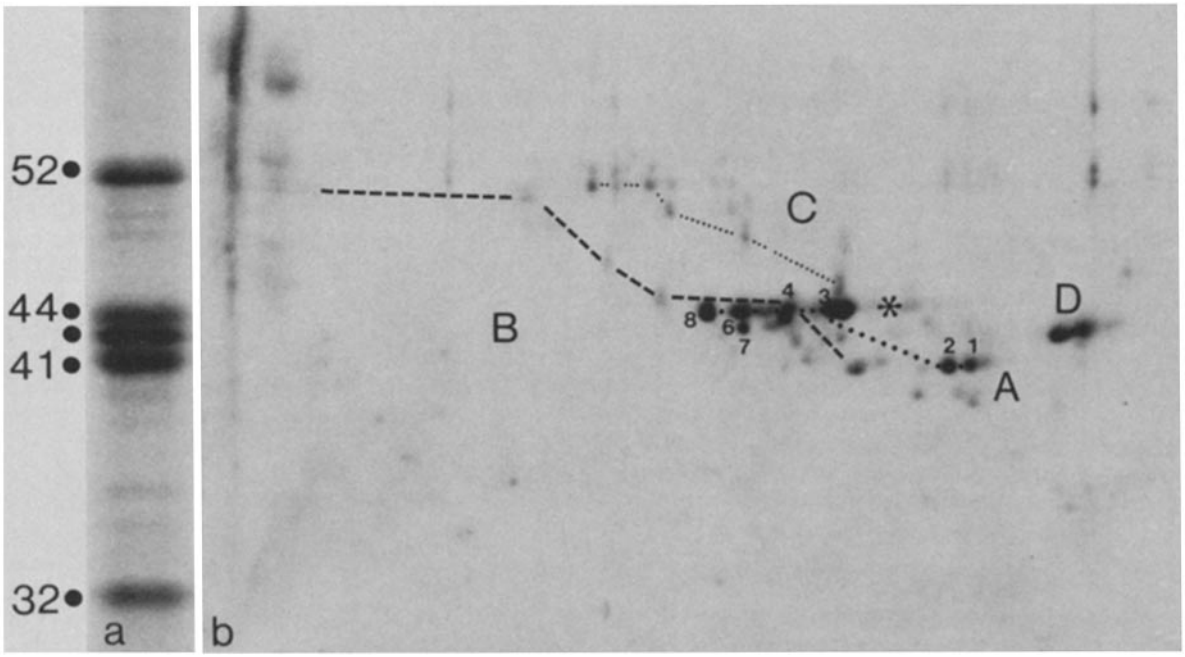

FIGURE 6 Detail of (*a*) 1-D and (*b*) 2-D gels of PtK_1 cytoskeletal extracts concentrating on the 40,000–55,000 mol wt range. From the data in Fig. 5 the major spots are connected by their binding of different mAbs to form four series: *A*, *B*, *C*, and *D*. The position of chicken gizzard actin when run with these preparations is indicated (*); pH ranges from about 5 (*right*) to 7 (*left*).

TABLE I

Properties of Six Monoclonal Antibodies Raised against PtK_1 Cytoskeleton Extracts

	Monoclonal antibody serial number					
	LE61	LE62	LE63	LE64	LE65	LE41
Immunoglobulin classification	IgG1	IgG1	IgG1	IgG2a	IgG2a	IgG1
Immunoglobulin yield in tissue culture ($\mu g/ml$)				30	80	
Immunofluorescence titre of culture supernatants	1/64	1/40	1/40	1/32	1/64	1/64
Spot series (Fig. 6) stained on 2D gels	A	A	B	D	A	C
	Binding to cells by immunofluorescence					
PtK_1 (antigen) and PtK_2 cell lines in tissue culture	++	++	++	++	++	++
Frozen sections of rodent tissues:						
(a) *Simple epithelia*						
respiratory (trachea, bronchioles, alveoli)	++	–	–	–	++	–
mammary gland epithelium	++	–	–	–	++	–
thymus epithelium	++	–	–	–	++	–
liver bile ducts	++	–	–	–	+	–
intestinal epithelium	+	–	–	–	+	–
kidney: collecting tubules	++	–	–	–	++	++
bladder epithelium	++	–	–	–	+	–
(b) *Stratified squamous epithelia*						
epidermis: keratinocytes	–	–	–	–	–	–
tongue epithelium: keratinocytes	–	–	–	–	–	–
tongue epithelium: taste bud cells	++	–	–	–	++	–
oesophageal epithelium	–	–	–	–	–	–
(c) *Non-epithelial tissues*						
connective tissue stroma	–	–	–	–	–	–
smooth, cardiac and striated muscle	–	–	–	–	–	–
nervous tissue: spinal cord	–	–	–	–	–	–

All immunofluorescence data were collected by evaluating the test preparations against positive control (sections stained with mAb giving a different pattern) and negative control (sections stained with 2nd antibody only) preparations. The staining of frozen sections was carried out on unfixed sections of both rat and mouse tissues in all cases above.

the lower mol wt polypeptides within the range (2, 11). The 32,000 mol wt band was very variable and disappeared when an attempt was made to remove the DNA-containing material from the sample. (Overloading the gels revealed many minor components in the antigen preparations, and this was reflected in the appearance of other lines, not described here, making antibodies to various components such as nuclear envelope lamin proteins and extracellular matrix material.) Immunoau-

toradiography of SDS gels showed that five of the monoclonal antibodies each recognized more than one gel band; the sixth one, LE64, recognized only one band of the 41–45,000-mol wt group. In PtK_1 cell samples all the antibodies recognized some of the 41–44kd polypeptides; two (LE41 and LE63) recognized material in the 50–55,000-mol wt range and three (LE41, LE62, and LE65) also recognized their determinant in minor components within the 25–30,000-mol wt range. There were also polypeptides that were recognized by LE61 and LE65 in similar cytoskeleton preparations from HeLa, BS-C-1 and CV-1 cells, and these were also within the 40–45,000 mol wt range. HeLa cells have already been shown to contain keratin-related determinants (7).

To achieve greater resolution around this crowded region of the gel, the immunoautoradiography technique was repeated on two-dimensional gels with emphasis on the polypeptides in the 40–45,000 mol wt range (Fig. 5). The 1-dimensional bands of this region were then resolved into a complex cluster of spots seen with Coomassie Blue staining which focus with pI values between pH 5 and 6 and show a marked trend towards increase in pI value with increase in molecular weight.

The autoradiographs of the 2-dimensional gels confirm that each mAb recognizes more than one polypeptide, and the distribution of spots sharing the same determinant also tends to follow a "bigger-to-basic" trend. By carefully aligning and superimposing tracings of the autoradiographs on the corresponding Coomassie Blue-stained gels (outlines in Fig. 5) it can be seen that no two monoclonal antibodies are directed against exactly the same determinant, because each mAb has its own individual "fingerprint" pattern of binding site homologies. It should be noted that whether or not the multiple spots are degradation products is irrelevant to the distinction of each mAb by its own binding pattern. The information from Fig. 5 is summarized in Fig. 6. The principal Coomassie Blue-stained spots can be grouped together according to their shared mAb binding sites into four series, A, B, C, and D. At this resolution, three of the mAbs (LE61, LE62, and LE65) bind to spots within the series designated as A, and LE64 stains only the pair of D spots, seen as a single band of around 43,000 mol wt in the 1-dimensional gels. Binding of all the mAbs is notably excluded from one major spot of 43–44,000 mol wt which lies close on the acidic side of A_3, to the basic side of the chicken gizzard actin control, and whose identity has not yet been ascertained but could be related to actin of PtK_1 cells.

The subtle immunofluorescence differences reported above between some mAbs on PtK_1 cells is confirmed by clear differences between the corresponding antibody distributions on the gels. The uniqueness of LE64 is most striking; the acidic pair of spots of the D series are not in fact picked up by any of the other antibodies. They lie away from the "bigger-to-basic" trend and are not accompanied by the usual scatter of spots along a parallel diagonal, and taken with the immunofluorescence data this suggests that this component of PtK_1 tonofilaments may be of a rather different chemical nature from the others.

One can also see from these gels that the two widely cross-reactive antibodies are the only ones to recognize A_1, A_2, and A_3, so these spots may indicate the location of the simple epithelium antigenic determinant. Material in the region of A_4 on the other hand is bound by four out of six of the mAbs, (not by LE64 or LE41), whereas binding to spots A_6, A_7, and A_8 distinguishes the antigenic determinant of LE61 (positive) from LE65 (negative).

DISCUSSION

The monoclonal antibodies described here can be regarded as specific markers for epithelial cells and, within these cells, for the intermediate filament subclass peculiar to epithelia, the tonofilaments or cytokeratin filaments, because they all failed to react with any structure in any of the nonepithelial mammalian cells tested. The use of a group of monoclonal antibodies which are directed against different aspects of the same cellular structures provides information about the organization of the filaments within the cell, as well as about the relationship of the antigenic determinants to epithelial differentiation.

That the proteins which these mAbs recognize belong to the group regarded as cytokeratins by other workers is indicated by: (*a*) the immunofluorescence staining pattern in PtK and other epithelial cells, (*b*) the molecular weight range, and (*c*) the size-charge relationship of the spots to which the antibodies bind on gels. All the gel samples were of detergent-resistant material, and detergent resistance is another characteristic of intermediate filaments. It is, however, possible that some of these components are "associated" proteins, rather than subunits of a filament core: we know very little about intermediate filament associated proteins. The determinants described here are nevertheless consistantly, and specifically, associated with tonofilament structures in epithelial cells.

Distribution of mAb-defined Determinants within PtK_1 Cells

The fact that one (LE64) of the six mAbs appears to react only with PtK cells and that another (LE41) appears to have a high affinity for a kidney epithelium determinant across species shows a degree of restriction of tonofilament antibodies which has not been demonstrated so far with conventional antisera, although it is not unexpected. All six antibodies are shown to recognize different antigenic sites in PtK_1 filaments by their unique antibody "fingerprints" on 2-dimensional gels, although it is difficult to resolve these differences satisfactorily by immunofluorescence alone. The combination of the two techniques of immunofluorescence and immunoautoradiography to analyze the specificities of monoclonal antibodies to closely related antigens has proved to be extremely valuable; the high sensitivity and information content of the 2-dimensional immunoautoradiography has great potential. Within a single cell type, this collection of distinct mAb specificities provides information about relationships between tonofilament polypeptides that is complementary to, and compatible with, data already obtained on molecular similarities among keratin-related filaments by other methods. Pronounced similarities in amino acid composition and tryptic peptide digests have been found between tonofilament polypeptides from various sources (11), and evidence exists that these proteins are the multiple products of a family of genes (14).

Because each of the mAb reagents consists of a single immunoglobulin molecular species, with one kind of binding site to one antigenic determinant of a protein, cross-reactivity indicates a high and very localized degree of molecular similarity, which is not necessarily the case for whole antisera. The observation of shared determinants among polypeptides of different mol wt can be explained in terms of Steinert's model of alpha-keratin filament subunit structure (28). This proposes the arrangement of three polypeptide chains aligned side by side consisting of two regions of coiled-coil alpha-helices which are highly conserved, interspersed with highly variable, enzy-

matically vulnerable, nonhelical regions. If a similar structure holds for all keratin-related filament subunits, then the mAbs which recognize multiple polypeptides may be interpreted as recognizing determinants lying within the constant alpha-helical regions. If this is so, then there are clearly several classes of constant region structures in PtK cells.

In the PtK_1 preparations some groups of these related structures lie along diagonals in the two-dimensional gel of "bigger-to-basic" increases in molecular weight with charge. Another diagonal shift among tonofilament polypeptides, in mouse epithelia, appears to be related to the growth and transformation state of the cells (29). The staining of multiple spots by a monoclonal antibody that is almost certainly against tonofilaments has also been illustrated by Brûlet et al. (30), and again the spread of these spots shows a diagonal "bigger-to-basic" tendency. Some of these shifts may be due to phosphorylation, which is known to take place among tonofilament polypeptides (2). There is no evidence so far that significant glycosylation occurs (11). It remains to be seen whether the spots along the diagnonals are also the products of independent genes but it seems likely that within one series the spots may result from post-translational modifications.

Antisera with specificity for high (31) and low (11) molecular weight determinants in *stratum corneum* have been raised before, by immunization with appropriate SDS gel band material. The Fuchs and Green study (11) also describes a high mol wt-induced antiserum that recognized all the major bands. The use of monoclonal antibodies allows an even finer distinction of polypeptide relationships. The results presented here provide one explanation for serological cross-reactivity within tonofilaments: the antigenic material can be resolved into several series of related polypeptides which overlap each other in mol wt, each series defined by similar core particle structure. Except for those invloved in terminal keratinization, so far, no tonofilament accessory proteins have been recognized biochemically. With the electron microscope, however, it is easy to see nonfilamentous material associated with the lateral adhesions of tonofilaments in all locations, both as small cross-bridges (e.g. 32) and as large accumulations of dense cement-like material (e.g. 33). It has been suspected for some time that such filament-associated material may account for part of the heterogeneity seen on SDS gels of tonofilament preparations, and this is one possible explanation for the anomalous D spots (Fig. 6) recognized by mAb LE64.

Tissue Distribution of mAb-defined Interspecific Determinants

The antigens recognized by the two cross-reactive mAbs are specific to epithelia, but they are not detected in all types of epithelia. The determinant defined by the binding of mAb LE61 and probably also recognized by LE65, may be designated a simple epithelium antigen, because it has been detected in all of the nonsquamous internal lining epithelia so far assayed in frozen sections from rat and mouse, and human material (not shown here). The detection of this antigen can apparently be used to discriminate clearly between cells from simple epithelia and the (normal) keratinocyte cell type of stratified squamous epithelia. Data from the use of conventional prekeratin antisera (3) have not revealed this antigenic distinction so far.

A line of virally transformed keratinocytes, on the other hand, was found to express the simple epithelium antigen, as did the HeLa cell line, so that the genetic information responsible was evidently still available for activation; the antigen may even still be present in the epidermal cells but in a sequestered form which the mAbs cannot detect. The failure of the unfixed frozen sections of epidermis to stain with the antibodies was not due to penetration problems, as is demonstrated by Fig. 2; adjacent to unstained keratinocytes are brightly stained taste bud cells. Taste buds are secondary sense organs that arise from within the epidermis of the tongue and follow a differentiation pathway that is distinct from that of the keratinocytes.

The simple epithelium antigenic determinant (or determinants) is localized on components between 40 and 45,000 mol wt in HeLa, BS-C-1, CV-1, and PtK_1 cells (Fig. 4), and so is probably on a similarly low mol wt cytokeratin component in the virally transformed keratinocytes. The 2-dimensional gel immunoautoradiography specifically indicates the spots A_1 and A_2 at 41,000 mol wt and A_3 at 44,000 mol wt (Fig. 6) as the location of the determinants in PtK_1 cells. It should be pointed out, however, that this does not appear to be one of the small keratin polypeptides whose expression is lost gradually through the differentiating epidermis (34), because no indication has been found of its presence in the basal keratinocytes of adult epidermis. If it is lost secondarily, then its loss or sequestration must take place at an earlier stage in the epidermal commitment to squamous epithelial differentiation. The detection of the determinant in transformed keratinocytes and in rare basal cells in neonatal epidermis does suggest that it may be expressed in cells that are further removed from terminal keratinocyte differentiation than are normal adult basal cells, including the keratinocyte stem cells. The presence of the simple epithelium antigen in epidermis may denote a remnant embryonic cell type, or it may indicate another type of nonkeratinocyte in the epidermis. One obvious candidate for the latter is the innervated Merkel cell, the question of whose epidermal origin has been much discussed (35). It is not yet known whether or not there are any spontaneous keratinocyte-derived tumors which express these determinants, but, if this turns out to be the case, then the diagnostic value of a monoclonal antibody that can recognize such a change may be significant.

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REFERENCES

1. Fraser, R. D. B., T. M. MacRae, and G. E. Rogers. 1972. *In:* The keratins, their chemistry, structure, and biosynthesis. I. N. Kugelmass, editor. Charles C. Thomas. Springfield, Illinois.
2. Sun, T.-T., and H. Green. 1978. Keratin filaments of cultured human epidermal cells. *J. Biol. Chem.* 253:2053–2060.
3. Sun, T.-T., and H. Green. 1978. Immunofluorescence staining of keratin fibers in cultured cells. *Cell.* 14:469–476.
4. Matoltsy, A. G. 1975. Desmosomes, filaments, and keratohyaline granules: their role in the stabilization and keratinization of the epidermis. *J. Invest. Dermatol.* 65:127–142.
5. Skerrow, D. 1974. The structure of prekeratin. *Biochem. Biophys. Res. Commun.* 59:1311–1316.
6. Franke, W. W., K. Weber, M. Osborn, E. Schmid, and C. Freundenstein. 1978. Antibody to prekeratin. *Exp. Cell Res.* 116:429–445.

7. Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1979. HeLa cells contain intermediate-sized filaments of the prekeratin type. *Exp. Cell Res.* 118:95–109.
8. Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. *Nature* (*Lond.*). 283:249–256.
9. Lee, L. D., and H. P. Baden. 1976. Organization of the polypeptide chains in mammalian keratin. *Nature* (*Lond.*). 264:377–379.
10. Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1978. The intermediate sized filaments in rat kangaroo PtK2 cells. II. Structure and composition of the isolated filaments. *Cytobiologie.* 17:392–411.
11. Fuchs, E., and H. Green. 1978. The expression of keratin genes in epidermis and cultured epidermal cells. *Cell.* 15:887–897.
12. Sun, T.-T., C. Shih, and H. Green. 1979. Keratin cytoskeletons in epithelial cells of internal organs. *Proc. Natl. Acad. Sci. U. S. A.* 76:2813–2817.
13. Kemp, D. J. 1975. Unique and repetitive sequences in multiple genes for feather keratin. *Nature* (*Lond.*). 254:573–577.
14. Fuchs, E., and H. Green. 1979. Multiple keratins of cultured human epidermal cells are translated from different mRNA molecules. *Cell.* 17:573–582.
15. Köhler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumour lines by cell fusion. *Eur. J. Immunol.* 6:511–519.
16. Kennett, R. H., K. A. Denis, A. S. Tung, and N. R. Klinman. 1978. Hybrid plasmacytoma production: fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells, and human spleen cells. *In: Curr. Top. Microbiol. Immunol.* 81:77–91.
17. Shulman, M., C. D. Wilde, and G. Köhler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature* (*Lond.*). 276:269–270.
18. Lane, D. P., and E. B. Lane. 1981. A rapid antibody assay system for screening hybridoma cultures. *J. Immunol. Methods.* In press.
19. Kurki, P., E. Linder, I. Virtanen, and S. Stenman. 1977. Human smooth muscle autoantibodies reacting with intermediate (1,000) filaments. *Nature* (*Lond.*). 268:240–241.
20. Gordon, W. E., A. Bushnell, and K. Burridge. 1978. Characterization of the intermediate (10 nm) filaments of cultured cells using an autoimmune rabbit antiserum. *Cell.* 13:249–261.
21. Lawton, A. R., R. Asofsky, M. B. Hylton, and M. D. Cooper. 1972. Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to μ chain. *J. Exp. Med.* 135:277–297.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*). 277:680–685.
23. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
24. Burridge, K. 1978. Direct identification of specific glycoproteins and antigens in SDS gels. *In:* Methods in Enzymology, 50. S. P. Colowick and N. O. Kaplan, editors. Academic Press, New York. 54–64.
25. Osborn, M., W. W. Franke, and K. Weber. 1980. Direct demonstration of the presence of two immunologically distinct intermediate-sized filaments systems in the same cell by double immunofluorescence microscopy. *Exp. Cell Res.* 125:37–46.
26. Walen, K. H., and S. W. Brown. 1962. Chromosomes in a marsupial (*Potorous tridactylis*) tissue culture. *Nature* (*Lond.*). 194:406.
27. Taylor-Papadimitriou, J., E. B. Lane, I. McKay, and S. Chang. 1981. SV40 transformation of human keratinocytes. *J. Invest. Dermatol.* 76:425. (Abstr.).
28. Steinert, P. M. 1978. Structure of the three-chain unit of the bovine epidermal keratin filament. *J. Mol. Biol.* 123:49–70.
29. Winter, H., J. Schweizer, and K. Goerttler. 1980. Keratins as markers of malignancy in mouse tumours. *Carcinogenesis.* 1:391–398.
30. Brûlet, P., C. Babinet, R. Kemler, and F. Jacob. 1980. Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. *Proc. Natl. Acad. Sci. U. S. A.* 77:4113–4117.
31. Viac, J., M. J., Staquet, J. Thivolet, and C. Goujon. 1980. Experimental production of antibodies against stratum corneum keratin polypeptides. *Arch. Dermatol. Res.* 267:179–188.
32. Lane, E. B. and M. Whitear. 1980. Skein cells in lamprey epidermis. *Can. J. Zool.* 58:450–455.
33. Franke, W. W., C. Grund, M. Osborn, and K. Weber. 1978. The intermediate sized filaments in rat kangaroo PtK2 cells. I. Morphology in situ. *Cytobiologie.* 17:365–391.
34. Fuchs, E., and H. Green. 1980. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell.* 19:1033–1042.
35. Breathnach, A. S. 1980. The mammalian and avian Merkel cell. *Proc. Linn. Soc. Lond.* 9: 283–291.