

Proteins IEF (Isoelectric Focusing) 31 and IEF 46 Are Keratin-type Components of the Intermediate-sized Filaments: Keratins of Various Human Cultured Epithelial Cells

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ABSTRACT Mouse polyclonal antibodies have been raised against two human proteins (IEF [isoelectric focusing] 31, $M_r = 50,000$; IEF 46, $M_r = 43,500$) that have previously been shown to be present in HeLa cytoskeletons enriched in intermediate-sized filaments. Immunoprecipitation studies show that both proteins share common antigenic determinants with each other and with the putative human keratins IEF 36 and 44, also present in HeLa cytoskeletons. Indirect immunofluorescence studies showed that both antibodies revealed similar filamentous networks in various cultured epithelial cells of human origin. These included AMA (transformed amnion), HeLa (cervical carcinoma), normal amnion cells, Fl-amnion (transformed amnion), WISH-amnion (transformed amnion), Chang liver (liver), and Detroit-98 (sternal marrow). Human cells that did not react with both antibodies included skin fibroblasts, lung fibroblasts (WI-38), SV40-transformed lung fibroblasts, Molt 4 (leukemia), lymphocytes, and monocytes. These results were in complete agreement with the presence or absence of both proteins in two-dimensional gels of the different cell types. Exposure of AMA cells to demecolcine (24 h; 10 $\mu\text{g}/\text{ml}$) caused the total collapse of vimentin filaments but, as seen by indirect immunofluorescence, caused only a partial redistribution of the IEF 31 and 46 filaments. These results are taken to suggest that both proteins are components of the intermediate-sized filaments of the "keratin" type. The antibodies could be clearly differentiated by staining human bladder carcinoma E19 cells, as only the IEF 46 antibody stained a filamentous network in these cells. The occurrence of keratins IEF 31, 36, 44, and 46 in different cultured human epithelial cells has been studied using two-dimensional gel electrophoresis.

Epithelial cells including keratinocytes contain intermediate filaments (7–11-nm thick filaments) that are related to keratins of the epidermis as judged by cross-reactivity using various keratin antibodies (11, 13–17, 20, 23–26). Franke et al. (16) have proposed the term "cytokeratins" to describe the proteins that compose these filaments and have presented evidence for a diversity of these proteins in various epithelial cells and tissues (12). Recently, we showed that cytoplasmic skeletons from epithelial HeLa cells enriched in intermediate filaments exhibited, besides vimentin, a few proteins that were likely candidates for keratins. In this study we show that mouse poly-

clonal antibodies raised against human proteins IEF (isoelectric focusing) 31 ($M_r = 50,000$) and IEF 46 ($M_r = 43,500$ (HeLa catalogue numbering system; 3, 5) react with intermediate filaments of the "keratin" type in a variety of human cultured cells of epithelial origin. Using a sensitive immunoprecipitation assay, we further show that both keratins share common antigenic determinants with each other and with the putative human keratins IEF 36 and 44 (3, 5, 7, 8, 16). The occurrence of the four keratinlike proteins in different cultured human epithelial cells has been determined by two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Cells: All the cultured cells used in this study were free of mycoplasma and were grown in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin 50 μ g/ml). The secondary amnion cells and skin fibroblasts were a gift from the Department of Human Genetics of Aarhus University and were used between passages number 2 (p2) and 4 (p4). The EJ 19 cells (human bladder carcinoma) were a gift of Dr. L. M. Franks (18).

Antibody Production: The cell content of approximately 30 confluent Nunc flasks (250 ml) seeded with transformed human amnion cells (AMA) was used to prepare proteins for immunization. The cell monolayers were washed four times with sterile Hanks' and scraped off with a rubber policeman in a final volume of 10 ml of Hanks'. After centrifugation, the pelleted cells were treated with DNase and RNase (21), lyophilized, and dissolved in IEF lysis buffer (21). The procedure for two-dimensional gel electrophoresis (IEF) has been described in detail elsewhere (8, 21). Following electrophoresis of samples (mixed with tracer radioactive proteins), IEF 31 and 46 were located using the autoradiograms and cut from the unfixed dried gels. The protein was eluted from the gels by shaking overnight at room temperature in 1 ml of 0.1% SDS (9), lyophilized, and redissolved in 1 ml of sterile Hanks'. An equal volume of complete Freund's adjuvant was added to this solution. Two BALB/c mice were immunized intraperitoneally each with 0.3 ml of these suspensions (containing \sim 20 μ g of proteins) and the injections were repeated at 15-d intervals until a positive test was obtained as judged by immunofluorescence of methanol:acetone-fixed AMA cells. After a booster injection, the animals were bled from the heart. About 0.4

ml of sera was obtained in each case. The animals also produced \sim 1 ml of ascites fluid as a result of the repeated intraperitoneal injections. Both the sera and the ascites fluid yielded the same immunofluorescent patterns. All the experiments reported here, however, were carried out with the sera.

Immunoprecipitation: The procedure used was similar to that described by Blöse and Meltzer (1). All the modifications detailed below were introduced to decrease the background to a minimum (Fey, Mose-Larsen, and Celis, manuscript submitted for publication). AMA grown in monolayers and labeled with [35 S]methionine (2, 6, 10) were washed twice in situ with Hanks' buffer (room temperature), lysed with 25–50 μ l of SDS sample buffer (2% SDS, 2.5% Dextran T 500, 100 mM dithiothreitol [DDT], and 80 mM Tris-HCl pH 6.8), and heated for 5 min at 100°C. Even though the heating step caused modification of a few polypeptides, this treatment is essential to achieve complete solubilization of the sample. The cell extracts were then cooled on ice and diluted 1:20 with a solution containing 1% Triton X-100, and 1% sodium deoxycholate in Tris buffer (150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% sodium azide, pH 7.5). The final concentration of SDS was 0.1%. Approximately 60 mg of protein A-Sepharose Cl 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) were swollen per immunoprecipitation, washed five times in TDS buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS in Tris buffer), and resuspended to give a 10% suspension of the gel (\sim 200 μ l). Immediately before use, the cell extracts were clarified by centrifugation at 145,000 g for 2 h. Aliquots (0.5 ml) of the supernatant (sufficient for four immunoprecipitations) were preabsorbed three times (20 min each) with 20 μ l of nonimmunized mouse serum plus 200 μ l of protein A-Sepharose (10% suspension) and mixed in a test tube placed on a wheel rotating at 4 rpm and at an angle of

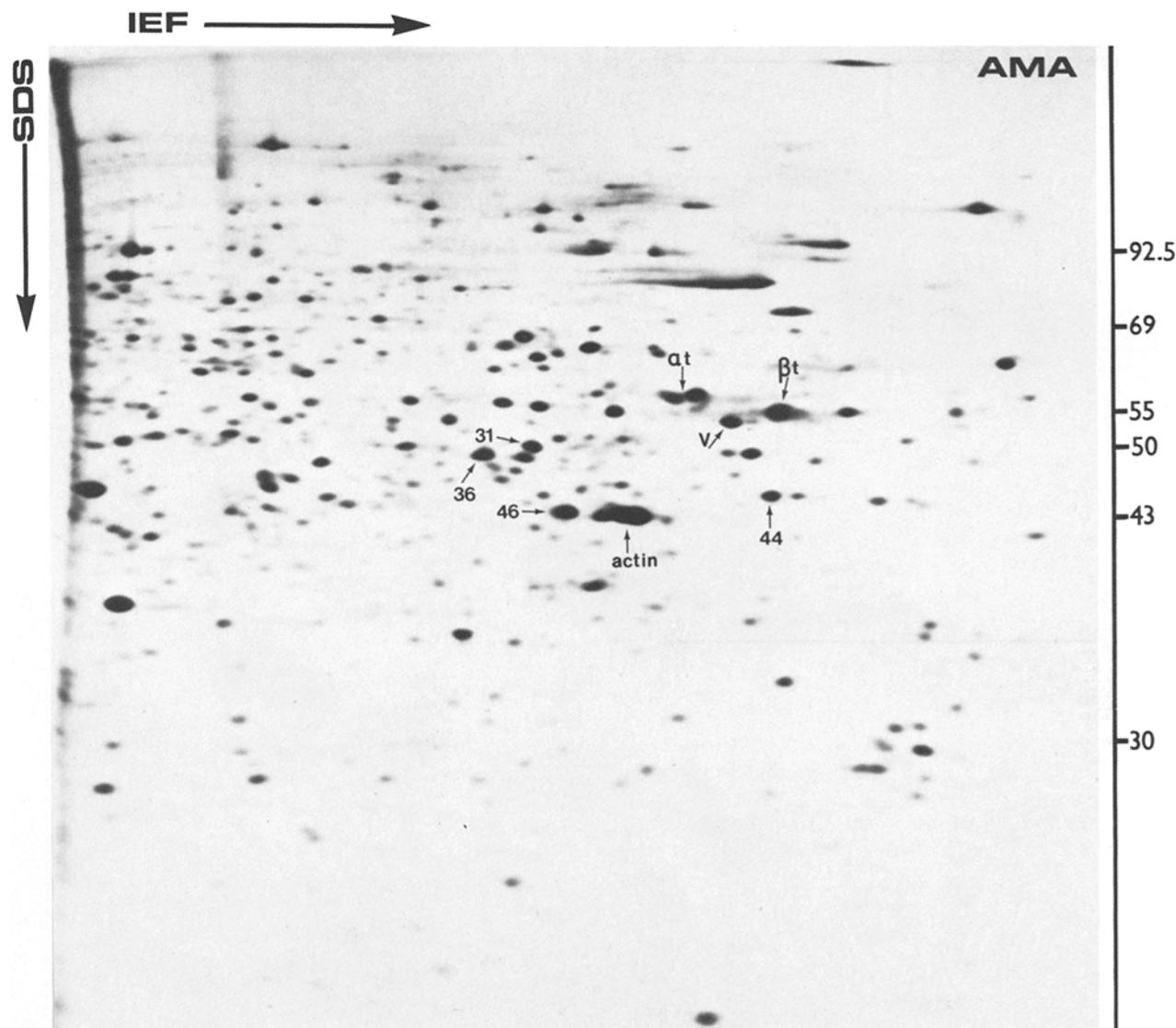


FIGURE 1 Fluorogram of an IEF two-dimensional gel of [14 C]amino acids labeled proteins from transformed human amnion cells. AMA (4). The cells were labeled for 16 h with a mixture of 16 [14 C]amino acids (Amersham Corp., Arlington Heights, IL; CBF 104) as previously described (3, 6). IEF 31, 36, 44, 46, α -tubulin (IEF 19), β -tubulin (IEF 25), vimentin (IEF 26) and total actin (IEF 47) are indicated for reference. The numbering system corresponds to that of the HeLa catalogue (3, 5). Molecular weights, $\times 10^{-3}$.

45°. The pelleted protein A-Sepharose was washed three times in TDS buffer and lyophilized. 2 μ l of the antibody (crude sera) were then added to 250 μ l of the preabsorbed extract and further incubated for 1 h. To this solution, 50 μ l of the protein A-Sepharose suspension was added, mixed for 30 min, and centrifuged. The pelleted protein A-Sepharose-antibody-antigen complex was washed three times and then lyophilized. This pellet, and those from the preabsorption steps, were extracted with 40 μ l of lysis buffer for IEF and NEPHGE (nonequilibrium

pH gradient electrophoresis) (21, 22). Protein A-Sepharose was removed by centrifugation. After electrophoresis and processing for fluorography (19), the gels were exposed to x-ray films for 5–10 d.

Indirect Immunofluorescence: Cells grown on glass cover slips (12 \times 12 mm) were washed twice with Hanks' and treated for 4 min at -20°C with absolute methanol, followed by a similar treatment with acetone. After washing extensively in Hanks', the cover slips were covered with 20 μ l of antibody (1:80 dilution in Hanks') and incubated for 45 min at 37°C in a humid environment. The cover slips were washed several times with Hanks' and covered with 20 μ l of FITC-conjugated rabbit anti mouse immunoglobins (DAKO, 1:160 in Hanks'). After 45-min incubation at 37°C in a humid environment, the cover slips were washed thoroughly with Hanks' and mounted in Gelvatol. Observations were made on a Zeiss photomicroscope equipped with fluorescence and phase-contrast optics.

Cell Labeling and Two-dimensional Gel Electrophoresis: The procedures of labeling cells with [^{35}S]methionine (2, 6, 10) or with a mixture of 16 [^{14}C]amino acids (3, 6) and of two-dimensional gel electrophoresis (8, 21, 22) have been described elsewhere.

RESULTS

Identification of IEF 31, 46, and Other Putative Keratins in Two-dimensional Gels of Human AMA Proteins

Fig. 1 shows a two-dimensional gel fluorogram (IEF) of total

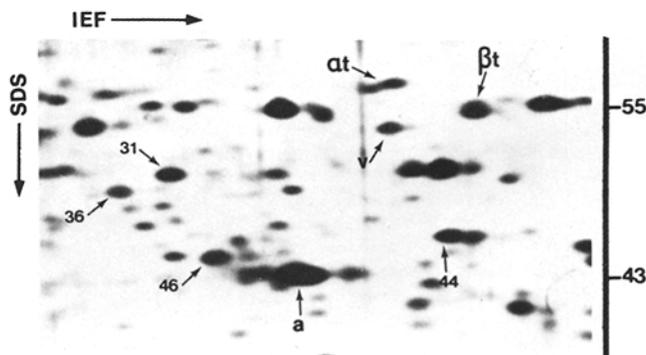


FIGURE 2 Two-dimensional gel electrophoresis (IEF) of proteins from AMA stained with silver nitrate (3). Only the relevant part of the gel is shown. Molecular weights, $\times 10^{-3}$.

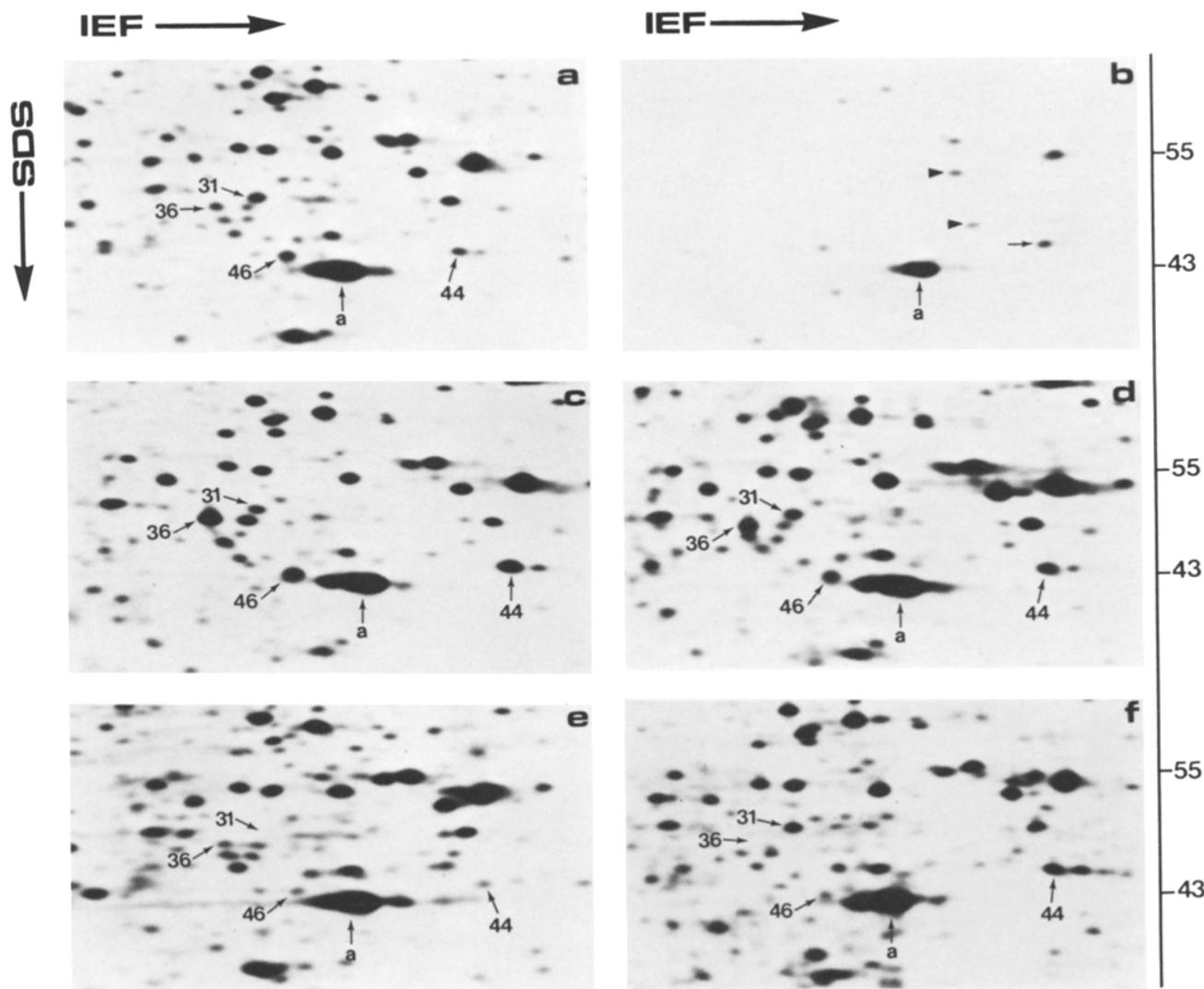


FIGURE 3 Two-dimensional gel electrophoresis (IEF) of [^{35}S]methionine-labeled proteins from various human cells. (a) HeLa, (b) epidermis, (c) WISH-amnion, (d) Fl-amnion, (e) E1 19, and (f) Detroit-98. The arrow in *b* indicates the epidermal keratin that comigrates with HeLa keratin IEF 44. Arrowheads indicate other keratins present in the epidermis. *a*, Actin. Molecular weights, $\times 10^{-3}$.

proteins isolated from transformed human amnion cells (AMA [4]) labeled with a mixture of 16 [¹⁴C]amino acids. The position of the putative keratins IEF 31 ($M_r = 50,000$), 36 ($M_r = 48,500$), 44 ($M_r = 44,000$) and 46 ($M_r = 43,500$) (3, 5, 7, 8) as well as of other major cytoarchitectural proteins, such as α - and β -tubulin

(α , β t), vimentin (v), and total actin (a), are indicated for reference (3, 15). Similar patterns were obtained when gels loaded with higher amounts of unlabeled protein were stained with silver nitrate (Fig. 2, only a fraction of the IEF gel is shown [3]). An identical set of putative keratins has so far been

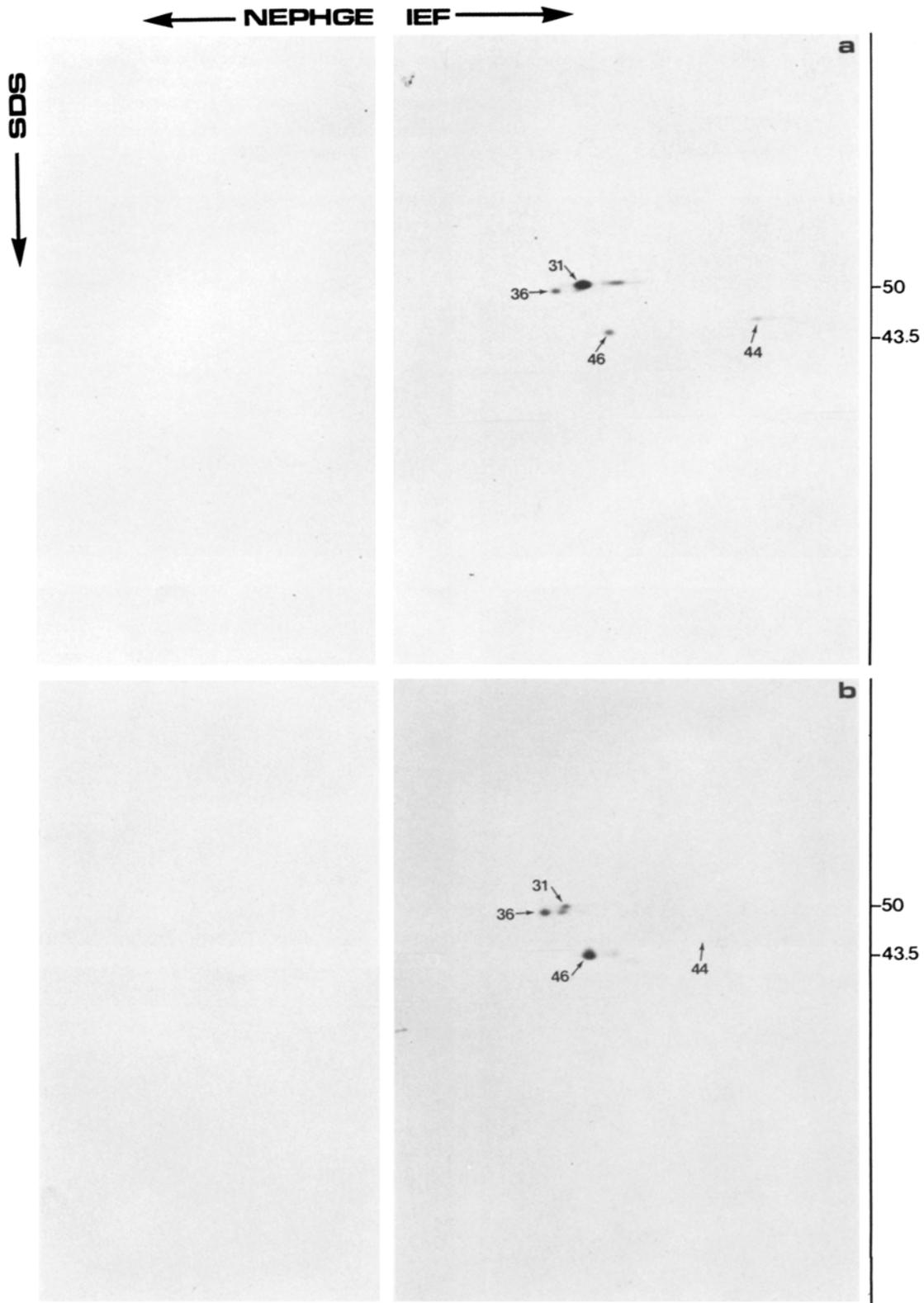


FIGURE 4 Characterization of antibodies. Total [³⁵S]methionine-labeled proteins from AMA cells were immunoprecipitated with the IEF 31 (a) and IEF 46 (b) antibody, and the immunoprecipitates were analyzed by two-dimensional gel electrophoresis. Molecular weights, $\times 10^{-3}$.

observed in the following human cultured epithelial cells: HeLa (cervical carcinoma, Fig. 3*a*) (3, 5, 7, 8), amnion (4), WISH-amnion (transformed amnion; Fig. 3*c*) FI-amnion (transformed amnion, Fig. 3*d*) and Chang liver (liver, not shown). The latter three cell lines are not contaminated by HeLa as shown by their two-dimensional protein patterns. Of the four putative keratins, only IEF 44 co-migrated with a human epidermal keratin as indicated with an arrow in Fig. 3*b*. Other acidic epidermal keratins are indicated with arrowheads in Fig. 3*b*.

Specificity of the Antibodies

Mouse polyclonal antibodies were raised against purified

proteins IEF 31 and 46 recovered from two-dimensional gels (see Materials and Methods) and their specificities were determined by a sensitive immunoprecipitation procedure (1; see also Materials and Methods). Fig. 4*a* and *b* show two-dimensional electrophoresis analyses (IEF, NEPHGE) of immunoprecipitates from total [³⁵S]methionine proteins from AMA cells reacted with the mouse polyclonal antibodies directed against proteins IEF 31 (Fig. 4*a*) and 46 (Fig. 4*b*), respectively. The IEF 31 antibody reacted mainly with protein 31, but it also showed significant cross-reactivity with the putative keratins IEF 36, 44, and 46 (Fig. 4*a*). Similarly, the IEF 46 antibody reacted mainly with IEF 46 but it also cross-reacted to different extents with proteins IEF 31, 44, and 46 (Fig. 4*b*).

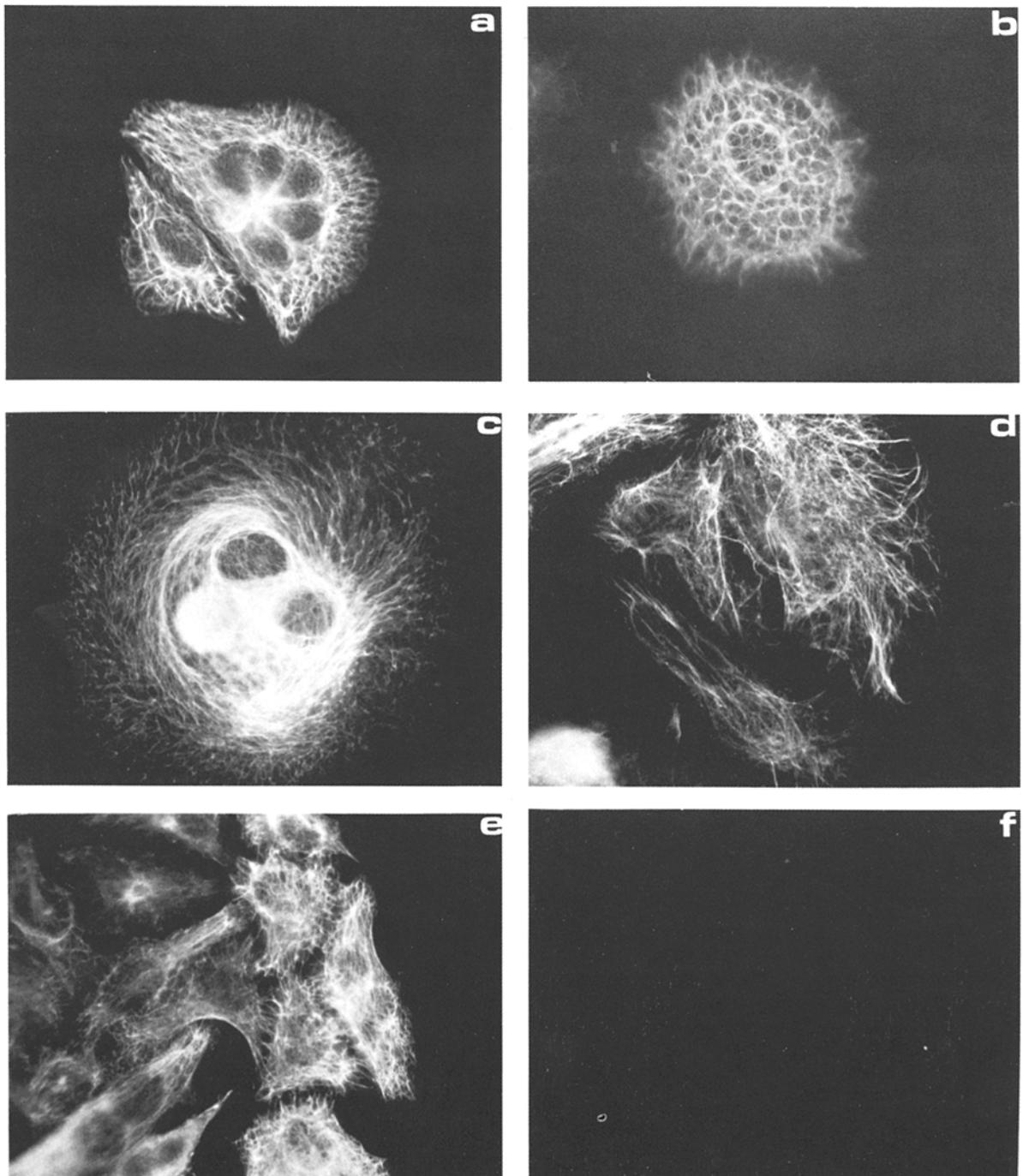


FIGURE 5 Indirect immunofluorescence of various human cells reacted with mouse anti IEF 31 (dilution = 1:80). (a-c) AMA; (d) normal amnion cells; (e) FI-amnion and (f) normal human skin fibroblasts (p4). $\times 320$.

Indirect Immunofluorescence

AMA (4) reacted with either IEF 31 or IEF 46 antibody and viewed by fluorescence microscopy, showed an extensive fila-

mentous network that extended throughout the cell (Figs. 5 *a-c* and 6 *a* and *b*). Normal human amnion cells which have these proteins (Fig. 7 *a*) also showed an extensive filamentous network (Figs. 5 *d* and 6 *c*). Human skin fibroblasts (p4) did

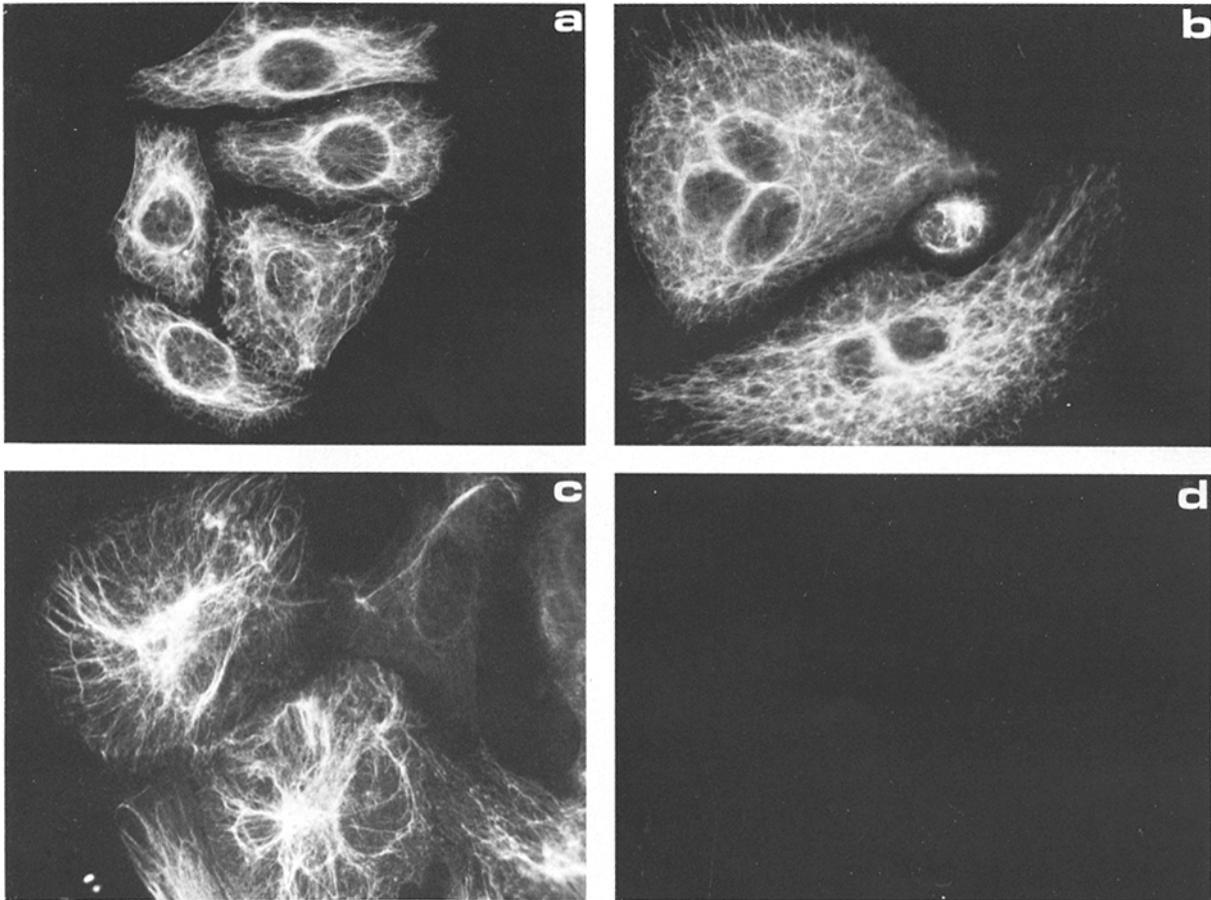


FIGURE 6 Indirect immunofluorescence of methanol:acetone-fixed human cells reacted with mouse anti IEF 46. (*a-b*) AMA, (*c*) normal amnion cells, and (*d*) human skin fibroblasts (p4). $\times 320$.

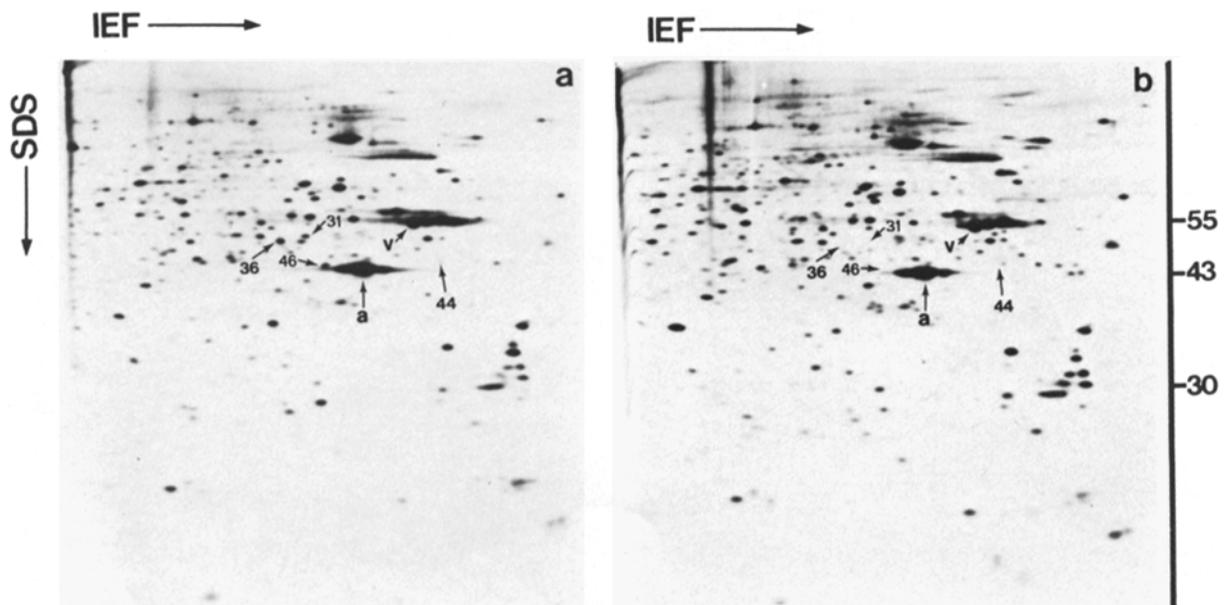


FIGURE 7 Two-dimensional gel electrophoresis (IEF) of [^{35}S]methionine-labeled polypeptides from (*a*) normal human amnion and (*b*) human skin fibroblasts. Both cell types were labeled for 16 h previous to electrophoresis. The position of vimentin (*v*) and actin (*a*) is indicated in both gels for reference. The skin fibroblasts have a protein migrating close to IEF 44, but it is not a keratin. Molecular weights, $\times 10^{-3}$.

not react with either antibody (Figs. 5*f* and 6*d*), in agreement with the absence of these proteins in [³⁵S]methionine-labeled (Fig. 7*b*) or silver-stained gels (not shown). Other human cell lines that reacted positively with both antibodies were HeLa, Chang liver, Fl-amnion (Fig. 5*e*), WISH-amnion, and Detroit-98 (sternal marrow) (results not shown). All these cell lines contain both IEF 31 and 46 as determined by two-dimensional gel electrophoresis (Fig. 3*a, c, d, and f*) (3–5). Human cells that did not react with either antibody and that lacked both proteins as judged by two-dimensional gel electrophoresis included human skin fibroblasts (Fig. 7*b*), SV40-transformed lung fibroblasts (4), Molt 4 (leukemia), lymphocytes, and monocytes.

Both IEF 31 and 46 antibodies could be clearly differentiated by staining methanol:acetone-fixed human bladder carcinoma cells (EJ 19) (Fig. 8*a and b*). While the IEF 46 antibody reacted with these cells to reveal a filamentous network (Fig. 8*a*), the IEF 31 antibody failed to give a positive reaction (Fig. 8*b*). These results are in agreement with the absence of IEF 31 in [³⁵S]methionine-labeled samples examined by two-dimensional gel electrophoresis (Fig. 3*e*).

Given the crossreactivity of the IEF 31 antisera with both IEF 46 and 44, it was interesting that we did not detect any significant staining of the EJ 19 cells with the IEF 31 antibody (Fig. 8*b*). This observation, however, may not be surprising, if one considers that for immunofluorescence we use

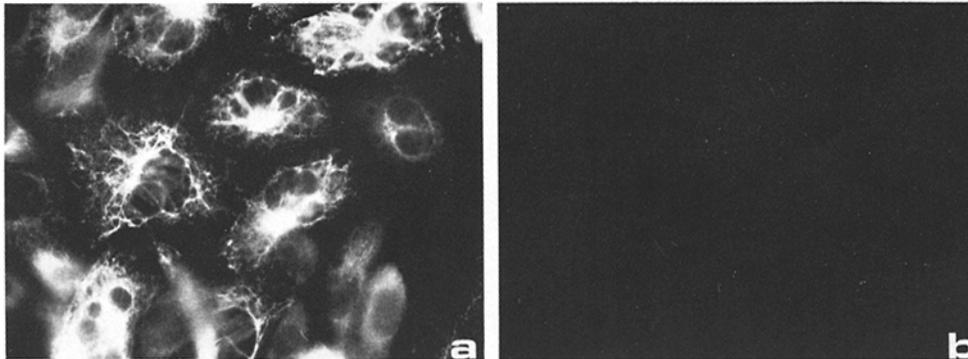


FIGURE 8 Indirect immunofluorescence of methanol:acetone-fixed EJ 19 cells (human bladder carcinoma) reacted with IEF 46 (*a*) and IEF 31 (*b*) antibodies, respectively. The intensity of the staining in *a* varied from cell to cell. $\times 320$.

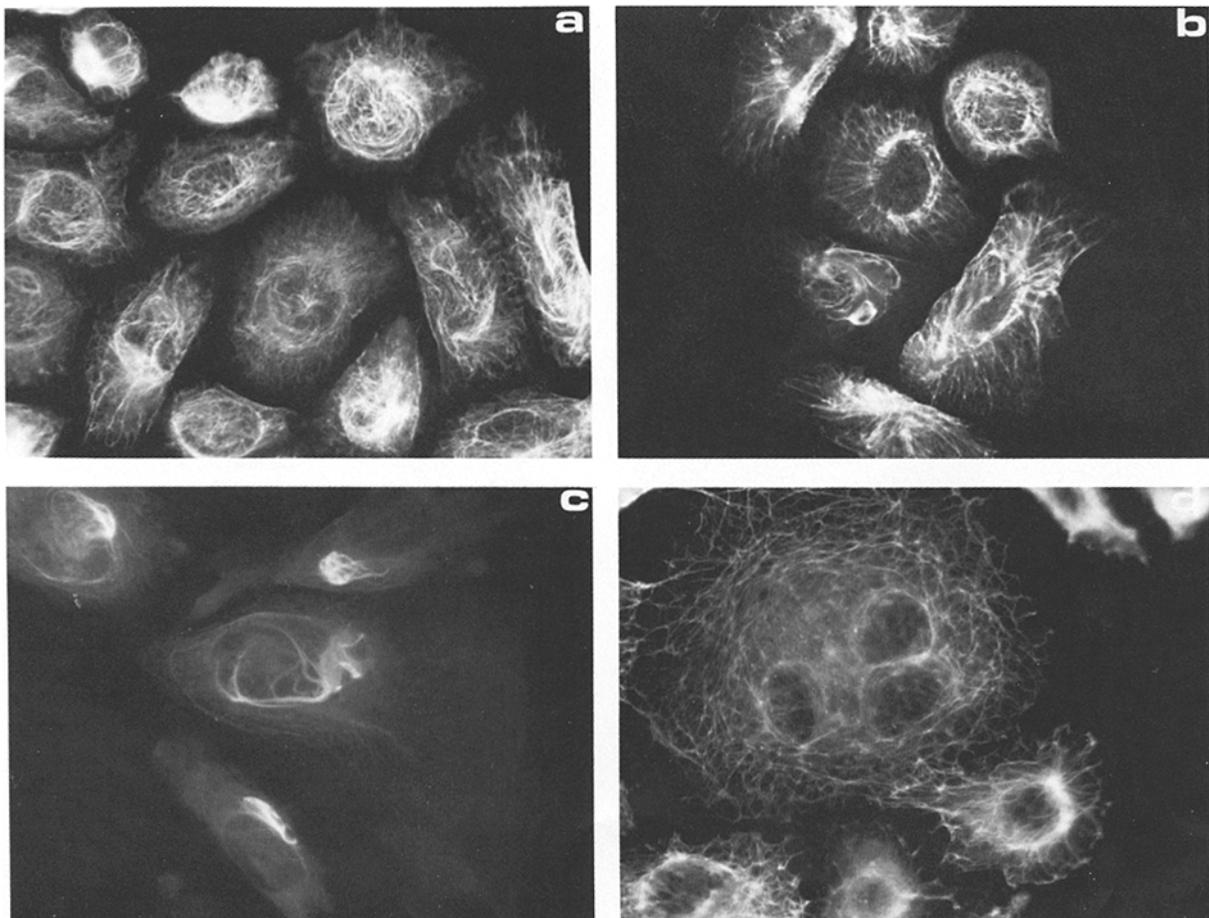


FIGURE 9 Indirect immunofluorescence of AMA cells treated with demecolcine or cytochalasin B. (*a, b, and c*) cells treated with 10 $\mu\text{g}/\text{ml}$ of demecolcine for 24 h. (*a*) AMA cells reacted with anti IEF 31 antibodies, (*b*) AMA cells reacted with anti IEF 46 antibodies, (*c*) AMA cells reacted with anti vimentin antibodies (a gift from Dr. S. Blose through Dr. J. V. Small), (*d*) AMA cells treated with 10 $\mu\text{g}/\text{ml}$ of cytochalasin B for 1 h and reacted with antibodies against IEF 31. $\times 320$.

methanol:acetone fixation previous to reaction with the antibody, a step not used for immunoprecipitation. It should also be mentioned that both antibodies do not react with formaldehyde-fixed cytoskeletons, and this suggests that they are sensitive to the fixative used.

Proteins IEF 31 and IEF 46 Are Components of the Intermediate-sized Filaments of the Keratin Type

Exposure of AMA to demecolcine (10 µg/ml; 24 h) resulted in a partial collapse of the IEF 31- and IEF 46-containing filaments (Fig. 9a and b). However, ~10% of the cell population showed perinuclear staining. In contrast, incubation of similarly treated cells with vimentin antibodies showed a distinctive perinuclear staining in virtually all cells examined (Fig. 9c). These results are taken to suggest that both IEF 31 and 46 are components of the intermediate-sized filaments of the "keratin" type. Exposure of transformed amnion cells (AMA) to Cytochalasin B (10 µg/ml) for 1 h did not result in a significant change in the distribution of the IEF 31- (Fig. 9d) or IEF 46-containing (not shown) filaments.

DISCUSSION

Evidence has been presented that human proteins IEF 31 and 46, which we have previously described as present in HeLa cell cytoskeletons enriched in intermediate-sized filaments (3, 7, 8), are components of the "keratin"-type filaments (16). These proteins share common antigenic determinants with each other and with the putative human keratins IEF 36 and 44 (3, 5, 7, 8). These four components seem to correspond to polypeptides 1 (M_r , ~54,000), 2 (M_r , ~52,000), 3 (M_r , ~48,000) and 4 (M_r , ~46,000) described by Franke et al. (12) in cytoskeletons of HeLa cells. These authors identified these proteins as prospective "cytokeratins" based on their solubility, cross-reactivity with keratin antibodies, and phosphorylation (12).

It is interesting to note that most of the human epithelial cultured cells analysed in this study, including HeLa (carcinoma of the cervix), AMA, normal amnion cells, Chang liver (liver), WISH-amnion (transformed amnion), and Fl-amnion (transformed amnion), exhibited all four keratins as judged by two-dimensional gel electrophoresis. Only Detroit 98 (bone marrow, lacks IEF 36; Fig. 3f) and EJ 19 (bladder carcinoma, lacks IEF 31) showed a simpler pattern of keratins. On the other hand, lymphocytes, monocytes, fibroblasts, and a leukemic cell (Molt 4) all lacked both proteins. These results are important in the light of the results of Franke et al. (12) which showed that the pattern of keratin polypeptides is characteristic for a specific tissue or cell type. Thus, it would seem possible that the presence of these keratins in the cell studies here may reflect different levels of differentiation of these cells. Further analysis of other epithelial cells will be needed to determine how widespread the occurrence of these keratins is in normal and transformed growing cells. Also, it would be important to determine the occurrence of these keratins in various human tissues in an effort to assess their putative value in characterizing neoplastic lesions or the origin of metastases (12).

At present, we do not know whether the keratins IEF 31 and 46 are components of the same filaments or whether they form independent filaments. From the results obtained with bladder carcinoma cells, however, it would seem that both keratins are

not necessary for the formation of the IEF 46-containing filaments. Experiments are now in progress to raise antibodies against the putative keratins IEF 36 and 44 in an effort to study their distribution in cultured cells and tissues.

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