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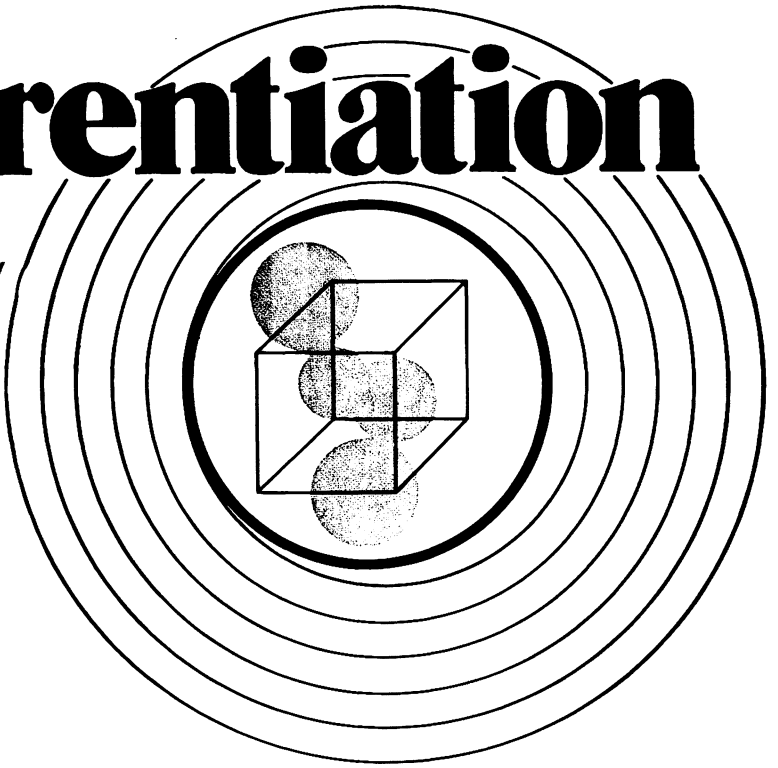

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Differentiation

Epithelial character and morphologic diversity of cell cultures from human amniotic fluids examined by immunofluorescence microscopy and gel electrophoresis of cytoskeletal proteins

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Abstract. In human fetuses of week 16 of pregnancy the various epithelia already contain intermediate-sized filaments of the cytokeratin type and desmoplakin-rich desmosomal plaques, as demonstrated by immunofluorescence microscopy of sections through frozen fetal tissues. When cells present in amniotic fluids obtained by amniocentesis during weeks 16–18 of pregnancy are allowed to grow in vitro, monolayer culture colonies of different morphology and cytoskeletal composition are obtained. We have examined such cells by electron microscopy and immunofluorescence microscopy, using antibodies to intermediate-filament proteins (cytokeratins, vimentin, desmin) and to desmoplakin, the major protein of the desmosomal plaque. Of the four major types of cell colonies regularly observed in such cultures, one morphotype (*ED*) is characterized by a cobblestone-like pattern of closely spaced, small cells which contain filaments stained with diverse cytokeratin antibodies, including those raised against epidermal prekeratin, as well as desmoplakin-positive sites at cell-to-cell-boundaries. In such colonies only a few individual cells have been detected which also express vimentin filaments. Colonies of morphotype *E* are formed by larger cells which often leave variously-spaced gaps between each other and contain filaments decorated by diverse cytokeratin antibodies as well as vimentin filaments but reveal desmosomal staining only in a certain subpopulation of cells. *AF*-colonies contain cells which do not grow in epithelial-like layers but in irregular arrays. These cells react with antibodies to cytokeratins and vimentin but are heterogeneous, even within the same colony, with respect to their reactions with certain antibodies to epidermal prekeratin and to desmoplakin. The fourth major type of colony is formed by elongated 'fibroblastoidal' cells (*F*-cells) which are stained by antibodies to vimentin and to some cytokeratins, but not by certain antibodies to epidermal prekeratins. *F*-cells do not reveal junctions stained by desmoplakin antibodies but do contain, like a certain proportion of *AF* cells, intracellular accumulations of desmoplakin-positive material. In cells of *E*-, *F*-, and *AF*-morphology double immunofluorescence microscopy has revealed bundles of intermediate-sized filaments stained with both antibodies to cytokeratins and antibodies to vimentin, besides other fibrils which are stained only with cytokeratin antibodies. Desmin filaments have not been detected in any of these colonies. Cells positive for vimentin but negative for all cytokeratin antibodies have only rarely been detected and are not regular components of such cultures. Gel electrophoretic analyses of cytoskeletal proteins of colonies of cell morphotypes have shown the presence of cytokeratins Nos. 7, 8, 18, and 19, together with some vimentin, in *E*, *AF*, and *F* colonies, but an absence of basic cytokeratin polypeptides. Desmin has not been detected.

These results emphasize the importance of non-morphologic markers in the identification and classification of cultured cells. Specifically, they show that all four major morphotypes of cell colonies which are routinely used for prenatal diagnosis consist of cells of epithelial origin and that, in normal fetuses, mesenchymally derived cells (fibroblasts, astrocytes, etc.) do not make a considerable contribution to such cultures. The different epithelial morphotypes which can be distinguished in such cultures could be due to their derivation from different epithelia or from cells of different degrees of differentiation in the same epithelium. An alternative explanation, which we consider more likely on the basis of the biochemically identical cytokeratin patterns, is that such morphotypes may represent different cell clones from the same epithelium varying in their response to the culture conditions. In general, culturing seems to promote in these cells the expression of vimentin filaments, in addition to cytokeratin filaments, and the reduction of desmosomes. In this sense, *ED* cells would be the most conservative in terms of maintenance of epithelial character, whereas *AF*- and *F*-cells are grossly altered to the extent that they are no longer readily identified as epithelial cells because of their altered morphology, their negative reactions with certain antibodies to some epidermal keratins, and the sparsity, if not absence, of desmosomes in many cells. The identification of the same cytokeratin polypeptides in all four morphotypes strongly suggests that the differences among the different cell types in their reactivity with different antibodies to cytokeratins do not reflect differences of expression of cytokeratins but rather differences of the arrangement of these cytokeratins in the filaments of the specific cell type. Possible fetal epithelia from which these cell colonies might have originated are discussed.

Introduction

Prenatal diagnosis at the cellular level is based mostly on in vitro cultures of cells derived from amniotic fluids ob-

tained during weeks 16–18 of pregnancy. Usually cells grown in monolayer colonies after two to three weeks of culturing are examined for possible karyotype and chromosomal aberrations, or metabolic and developmental disorders [for reviews see 31, 32, 55]. In such studies, identification and classification of the specific cells examined would be important for critical evaluation of these observations as well as for diagnosis and clinical advice. For example, the value of karyotype analysis in such cell cultures is overshadowed by cases of mosaicism or pseudomosaicism [38, 55] which theoretically could be due to the colonies grown in vitro originating from different fetal tissues, including the amnion epithelium. In addition, the identification of diverse cell types in such cultures could be of diagnostic value by itself, as demonstrated by the presence of astrocytes, detected by an acetylcholinesterase isoenzyme [e.g., 6, 63] and glia-type intermediate filaments, in cases of neural tube defects such as *spina bifida aperta* and anencephaly [1, 8, 70].

It has been observed by several authors that cell colonies grown from human amniotic fluids are morphologically not homogeneous, and diverse cell types have been distinguished by their specific growth patterns [29, 33; for review see 35], including cases of certain disorders such as neural tube defects (see above). Recently antibodies to proteins of intermediate-sized filaments have been introduced for the classification of cells according to their specific states of cell differentiation [4, 12, 15, 26, 36, 47–49, 53]. Epithelial cells grown in the tissue are characterized by the synthesis of filaments of the cytokeratin-type [12, 13, 56, 64, 65]; however, epithelial cells growing in vitro often begin to produce, in addition to cytokeratin filaments, also intermediate filaments of the vimentin type, normally not found in epithelial tissues [12, 14, 15, 18, 66, 68]. By contrast, intermediate filaments of the vimentin type are characteristic of mesenchymally derived cells and various other non-epithelial cells, including Sertoli cells of the testis, retinal cells, and eye lens-forming cells [3, 12, 16, 54]. Desmin filaments are typical of most types of muscle cells [46, 62; for reviews see 36, 47], whereas glial filaments have so far been found only in astrocytes and some other glial cells [48, 53, 59, 61, 72]. Neurofilaments are specific for neuronal cells [same references].

Using antibodies to epidermal prekeratin and immunofluorescence microscopy, it has previously been shown that amniotic fluid cell colonies of morphotype *E*, as defined by Hoehn and colleagues [33, 35], contain filaments of the cytokeratin type [7, 9]. Some colonies of other morphotypes, notably those classified as *F* and *AF* cells, have been negative with these antibodies [9], suggesting that they are either not derived from an epithelium or express a subset of cytokeratin polypeptides not recognized by the antibodies to epidermal prekeratin used in this study [for diversity of cytokeratin polypeptide expression in different epithelia see 20–22, 49, 67, 71]. In the same study the majority of cell colonies examined was found to be positively stained with vimentin antibodies [9]. Virtanen et al. [69] reported that most of the colony morphotypes grown in their cultures of amniotic fluid cells are positive with a certain antiserum to epidermal keratin as well as with antibodies to vimentin, from which they concluded that most of these amniotic fluid colonies are of epithelial origin.

Recently cytokeratin-specific antibodies, showing a broad range of cross-reaction between different polypeptides of the cytokeratin family of proteins have become available, both in the form of conventionally obtained antisera [e.g., 20] and as monoclonal antibodies [30]. Such antibody preparations allow the detection of most, if not all, types of epithelial cells and tissues. In addition, antibodies to constitutive proteins of the desmosomal plaque, the plasma membrane domain specific for cytokeratin filament attachment in epithelial cells, have been used as another nonmorphologic marker for the identification of epithelial cells [23, 25, 28, 51]. Therefore we have re-examined, using immunofluorescence microscopy and gel electrophoresis, the various morphotypes of cell colonies growing in cultures from amniotic fluids, in comparison with sections through frozen fetal samples from about the same period of gestation.

Methods

Amniotic cell cultures and tissues

Amniotic fluids were obtained by transabdominal amniocentesis during weeks 16–18 of pregnancy from women between 35 and 42 years of age, where amniocentesis was indicated. Conditions for cell culturing have been described [9]. The karyotype of these cell cultures was normal in all cases. Between days 9 and 15 of culturing, primary cell cultures were used for indirect immunofluorescence microscopy and preparations of cytoskeletal proteins.

Fetal tissue samples were obtained from three abortions induced for medical reasons (fetal nephrosis, trisomy 21, hysterectomy) in pregnancies of weeks 13–21 of pregnancy [cf. also 50]. Tissue samples included areas exposed to – or communicating with – the amniotic fluid, such as skin, trachea, tongue, esophagus, small intestine, colon, renal pelvis, and urinary bladder. Specimens were usually taken approximately 8 h after abortion and were frozen in isopentane cooled with liquid nitrogen to about $-150°$ C for 2 min and then stored at $-70°$ C until use. For immunofluorescence microscopy tissue pieces were mounted on cutting blocks and sectioned at $-20°$ C to $-30°$ C on a cryostat (Frigocut, model 2700; Jung, Nussloch, Federal Republic of Germany) into sections of 4–6 μm, using steel blades.

Gel electrophoresis of cytoskeletal proteins

Cell colonies grown on glass slides or coverslips were examined, still covered with culture medium, by phase contrast optics, and individual, selected, classified (*ED, E, AF, F*) colonies were marked by encircling the area occupied by the specific colony on the underside of the slide or coverslip, using a water-proof pen. Then the growth medium was replaced by phosphate-buffered saline (PBS), the cells were briefly dried with a hair drier, and the specific colony was once more encircled by a pen, this time on the upper surface. A drop of buffer was then placed over the selected and marked colony in such a way that the meniscus was only slightly larger than the diameter of the colony. The cells of the specific colony were then scratched from the substratum using a fine needle, suspended in buffer, and collected by drawing them up in a 1 ml syringe. Thereafter, the suspended cells were directly extracted and cytoskeletons were made and processed for two-dimensional gel electrophoresis as described previously [21, 22, 24, 49].

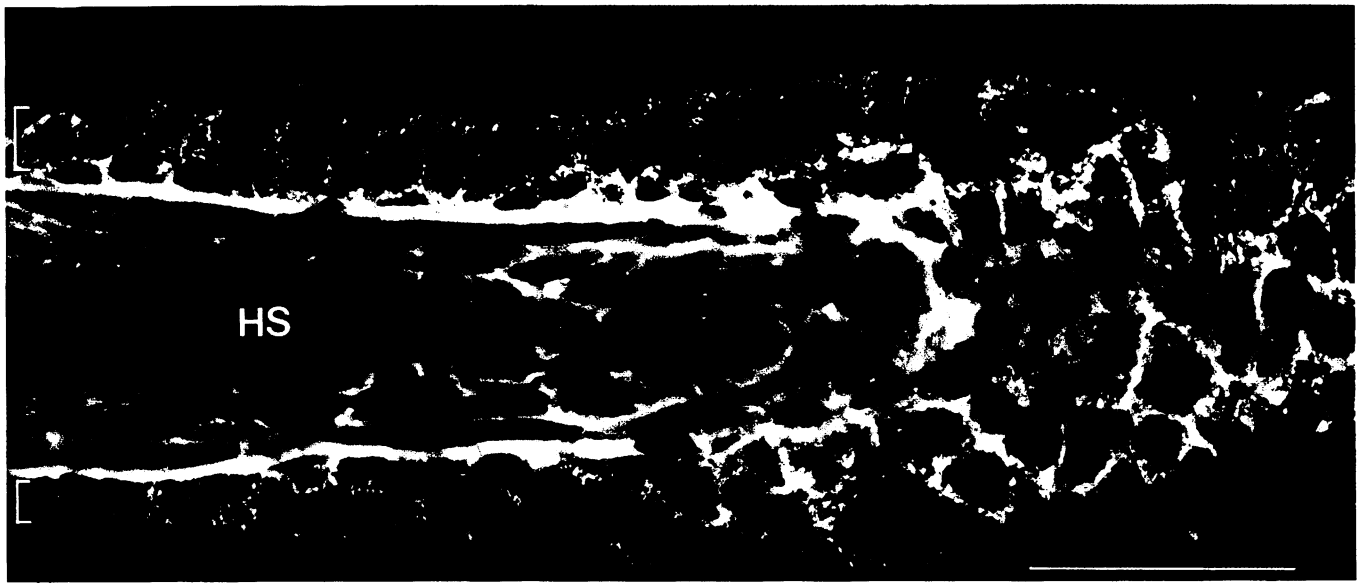

Fig. 1. Immunofluorescence microscopy of fetal skin (week 21) showing a fetal hair follicle after reaction with antibodies against desmoplakin. Note punctate desmosomal staining in outer hair root sheath epithelium. HS, hair shaft. *Bar* denotes 50 μm

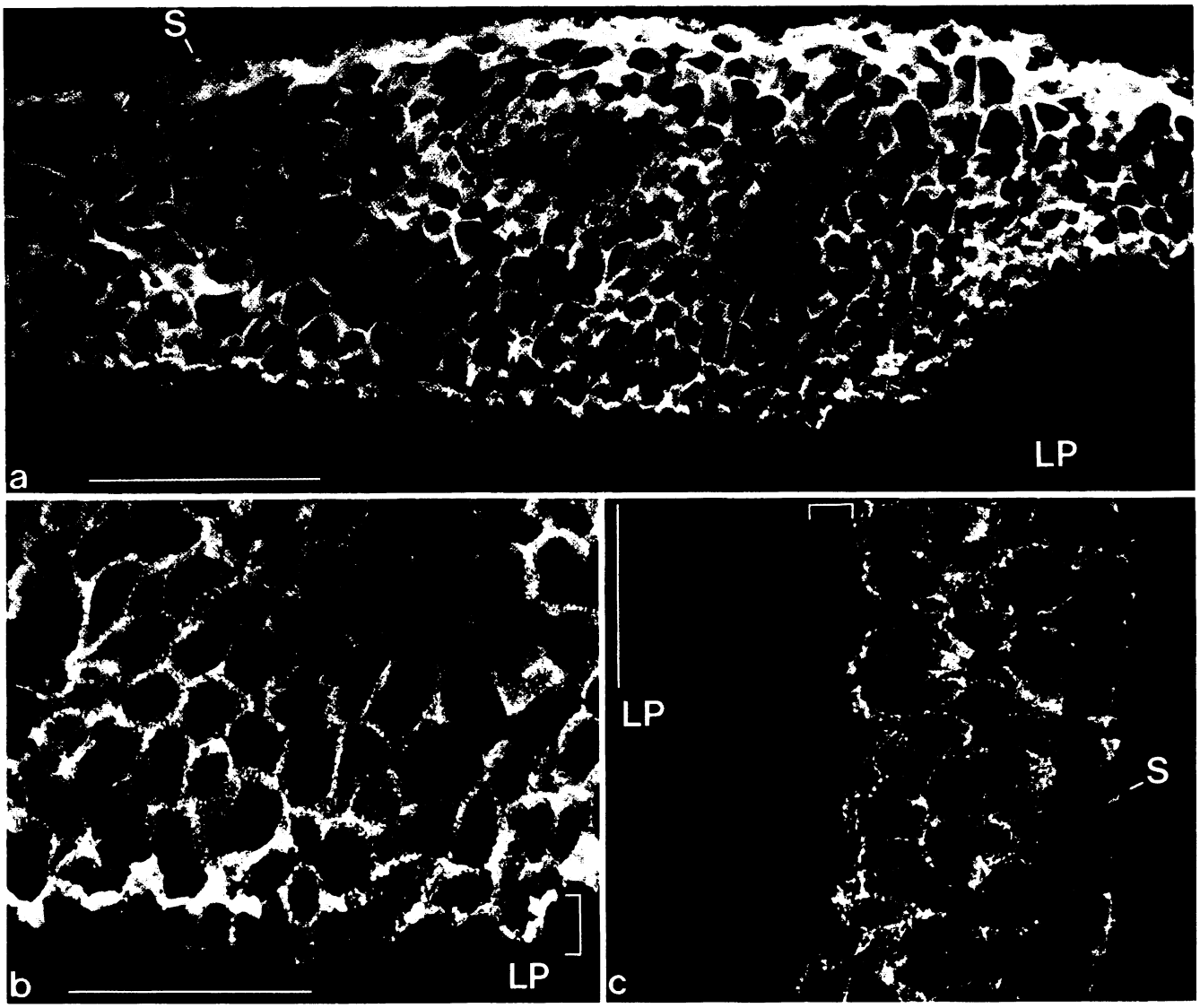

Fig. 2a–c. Immunofluorescence microscopy of fetal tongue **a, b** and esophagus **c** after reaction with antibodies to desmoplakin. The multilayered epithelia show distinct fluorescent points representing individual desmosomes. Note absence of staining in the *lamina propria* (LP). S, tissue surface. *Bars* denote 100 μm **a** and 50 μm **b, c**

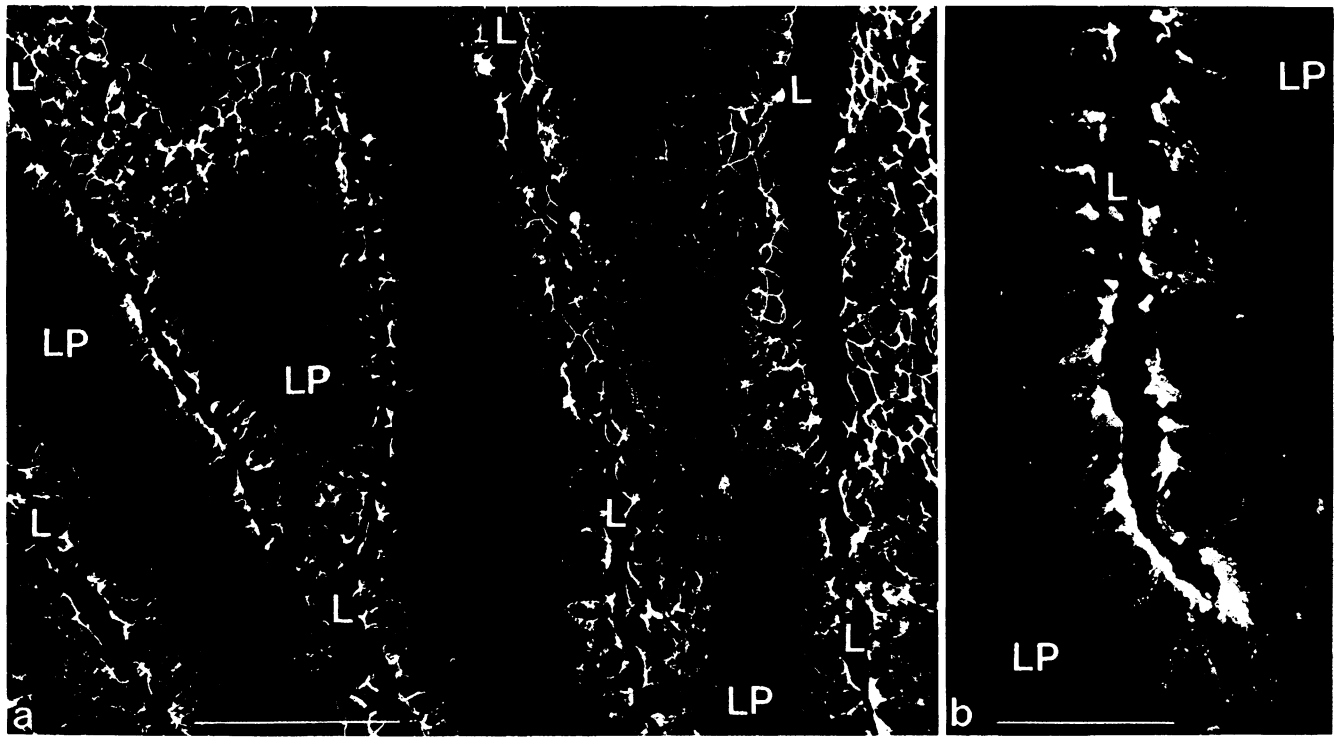

Fig. 3a, b. Immunofluorescence microscopy of the fetal jejunum after reaction with antibodies to desmoplakin. Note enrichment of desmosomal material in subapical regions (L, lumen). LP, *lamina propria*. *Bars* denote 100 μm **a** and 50 μm **b**

Antibodies and immunofluorescence microscopy

The following antibody preparations were used: (*i*) Guinea pig antibodies (IgG) against bovine epidermal prekeratin, reacting with prekeratin components I–VI of bovine muzzle [13, 19, 22]; (*ii*) Guinea pig antibodies (IgG) against murine liver cytokeratin D [20]; (*iii*) A monoclonal murine antibody (IgG2) known to react with an especially broad spectrum of diverse cytokeratin polypeptides (antibody K_G 8.13) [30]; (*iv*) A monoclonal antibody (IgM) reacting with cytokeratins Nos. 8 and 18 of human cytokeratin polypeptides (to be described elsewhere); (*v*) Guinea pig antibodies (IgG) against human vimentin [15]; (*vi*) Guinea pig antibodies (IgG) to bovine desmoplakin [25, 28, 51]; (*vii*) Guinea pig and rabbit antibodies specific for desmin [cf. 57].

For indirect immunofluorescence microscopy cell cultures were washed in PBS, fixed in methanol (−20° C) for 5 min, and then dipped 6 × 1 s into acetone (−20° C). The cultures then were air-dried, rinsed with PBS, and incubated with the first antibody for 30 min in 37° C. After washing in PBS (3 × 5 min) the second antibody was then added for 30 min at 37° C. The cover slips were washed again in PBS, mounted on glass slides, embedded with Mowiol, and observed in a photomicroscope III (Zeiss, Oberkochen, Federal Republic of Germany). Accessibility of antibodies could sometimes be improved by treating cell cultures grown on coverslips with 0.1% Triton X-100 (in PBS) for 1 min before fixing them in methanol. Sections of frozen tissues were air-dried and then processed for immunofluorescence microscopy as described above for cell cultures, except that fixation in methanol was omitted, and the sections were directly treated with acetone at −20° C for 10 min

For double label immunofluorescence microscopy, the two first antibodies (e.g., murine antibody K_G 8.13 and guinea pig antibodies to vimentin or antibody K_G 8.13 and guinea pig antibodies to desmoplakin) were applied immediately after each other (i.e., within 30–60 s). After washing (see above) the specific second antibodies (rhodamine-labeled goat antibodies to mouse IgG and FITC-labeled rabbit antibodies to guinea pig IgG) were added at brief intervals (30–60 s) and incubated as described for normal immunofluorescence microscopy.

Electron microscopy

Cells grown on coverslips were fixed, dehydrated, and embedded as described previously [23]. For immunoelectron microscopy, cell preparations were processed essentially as outlined by Kreis et al. [44].

Results

Immunolocalization of cytoskeletal proteins in fetal human tissues

Since early embryonic epithelia of the mouse, and probably also of other mammals, are characterized by very simple cytokeratin polypeptide compositions [26, 27, 37, 39, 40], it has been important to examine the state of differentiation of the various epithelia present in human fetuses of gestation weeks 16–21, i.e., the stages relevant for the study of amniocentetic samples including the usual 2–3 weeks of growth in vitro. Using immunofluorescence microscopy on cryostat sections through frozen fetal tissue, it can be shown that after week 16 of pregnancy the major epithelial organs of the fetus are already identifiable and positive for cytokeratins (data not shown; cf. Refs. 50, 52; S. Regauer, thesis, manuscript in preparation). At this stage, the epidermis al-

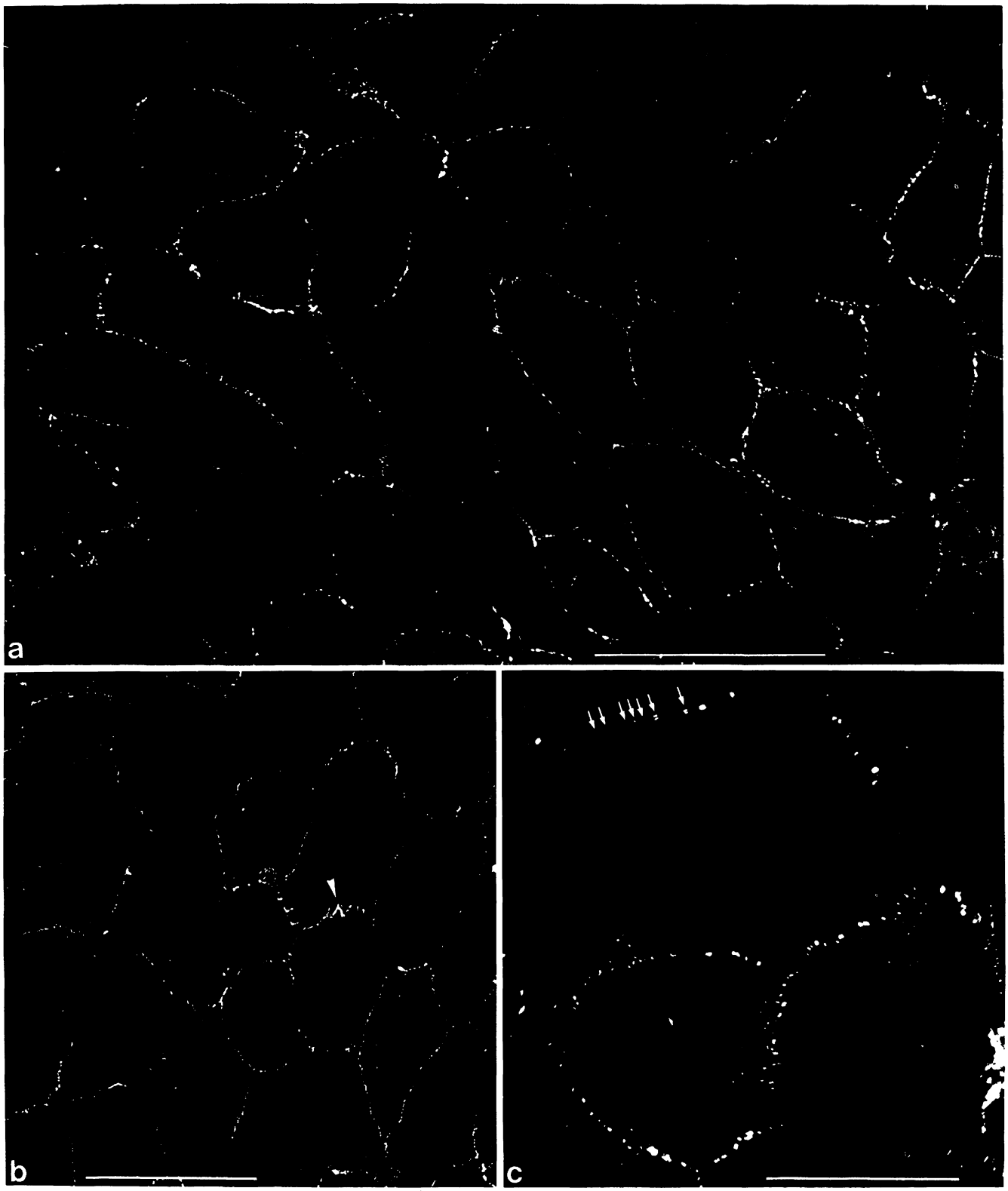

Fig. 4a–c. Immunofluorescence microscopy of cell culture from amniotic fluid samples, presenting a cell colony of morphotype ED after reaction with antibodies to desmoplakin. Note the typical cobblestone pattern with very close cell-to-cell attachments which are uniformly stained in dotted lines by these antibodies **a, b.** In certain regions, small cellular interdigitations are identified (one is denoted by an *arrowhead* in **b**). At higher magnification, occasionally the two plaques of each desmosome seem to be distinguished (e.g., *arrows* in the upper part of **c**). *Bars* denote 50 μm **a, b** and 20 μm **c**

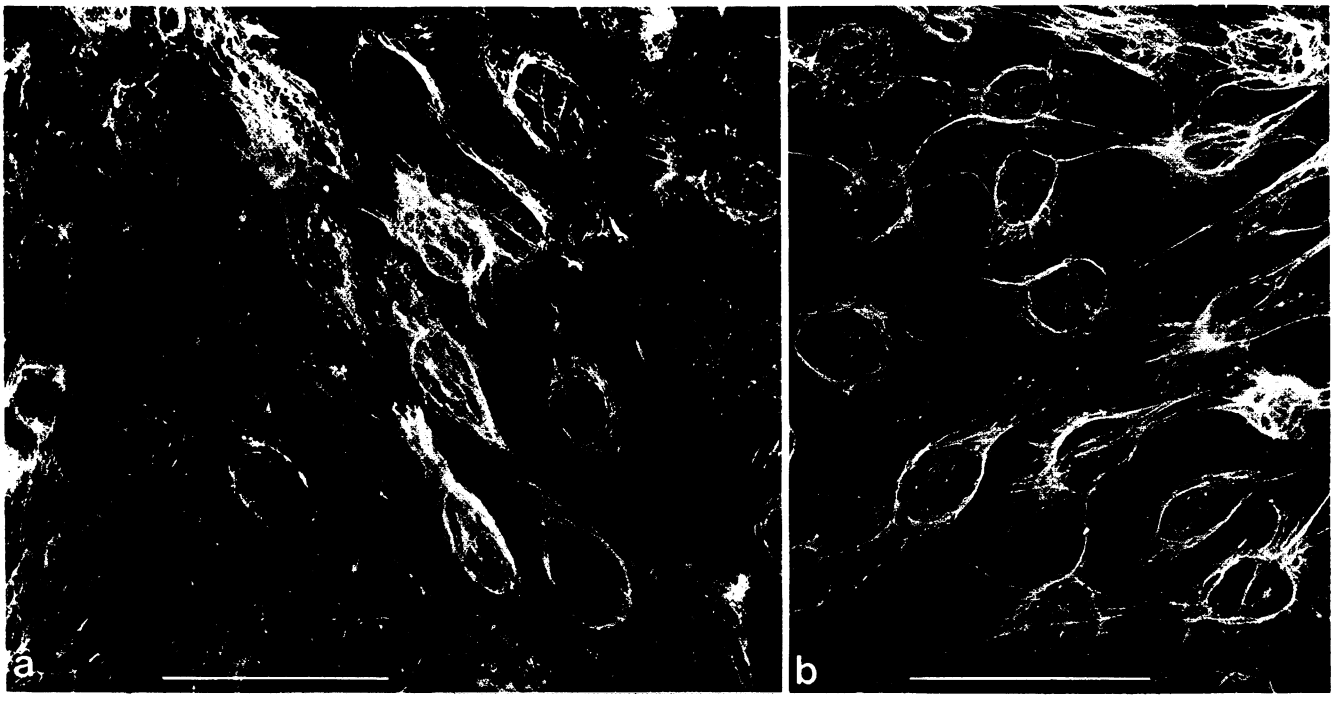

Fig. 5a, b. Immunofluorescence microscopy of cells of colonies of morphotype ED after reaction with **a** monoclonal antibody recognizing diverse cytokeratin polypeptides (K_G 8.13) and **b** antibodies against bovine epidermal prekeratin. Note corresponding fibrillar patterns with both antibody preparations, including typical tonofibril arrays. *Bars* denote 50 μm

ready contains 3–4 layers and peridermal cells have begun to desquamate into the amniotic fluid [cf. 50]. In addition we have used antibodies to desmoplakin in order to examine the presence and mode of distribution of this membrane-associated cytoskeletal protein. With these antibodies, epidermis (not shown) [cf. 50] as well as the outer epithelium of hair germs and developing hairs reveal the typical punctate staining patterns, reflecting the distribution of desmosomal structures (Fig. 1). Essentially similar arrays of desmosomes in high densities have been seen in other stratified epithelia such as mucosa of tongue (Fig. 2a, b) and esophagus (Fig. 2c). Moreover, internal organs also show the specific desmosomal arrangements (as an example the small intestine is shown in Fig. 3) as previously described for adult tissues [cf. 23, 25, 28]. No fetal epithelia positive for cytokeratins have revealed any significant reaction with antibodies to vimentin and desmin (data not shown). Amnion epithelium from similar fetal stages also shows a positive reaction with antibodies to cytokeratin and desmoplakin (an indistinct reaction with vimentin antibodies has also been observed in this epithelium, but its significance is difficult to assess) [9, 52].

Immunofluorescence microscopy of cultured cells

Cell cultures obtained from amniotic fluids grow in colonies which display a remarkable heterogeneity [35]. One very characteristic form of colony is represented by sheets of very small, nearly isodiametric cells, growing in a cobblestone-like pattern, which apparently are similar to the colonies classified as 'E-4 cells' by Virtanen et al. [69]. In these colonies the individual cells are demarcated by linear arrays of desmoplakin-positive dots, each dot representing a desmosome, a cluster of desmosomes, or a hemidesmosome (Fig. 4a–c). Occasionally, one can resolve two separate individual desmosomal plaques apparently within a given desmosome (*arrows* in Fig. 4c). Colonies of this type (designated morphotype *ED*, i.e., *e*pithelial and *d*ensely packed cells), which occur at highly variable frequencies in different cultures, have been positive for all cytokeratin antibodies examined, including the monoclonal antibody K_G 8.13 (Fig. 5a) and antisera against bovine epidermal prekeratin (Fig. 5b). They are negative with antibodies specific for desmin (not shown) and represent the only type of colony in which most of the cells are not stained with antibodies against vimentin (for detailed demonstration see below).

In our preparations, epithelial colonies of larger, but more loosely arranged cells, often with variably-sized 'gaps' between individual cells, have been much more frequently encountered and seem to correspond to the typical *E*-type colonies as defined by Hoehn and colleagues [33, 35]. While desmoplakin-positive dots at cell-to-cell boundaries, obviously representing normal desmosomes, are still common in such colonies (Fig. 6a, b), many of these cells also contain desmoplakin material deeper in the cytoplasm (Fig. 6a), often in punctuate patterns (Fig. 6b). Such intracytoplasmic, desmoplakin-positive structures might represent hemidesmosomes located at the bottom cell surface or endocytotically internalized desmosomal domains, similar to structures found in several other cell cultures [23, 43, 58, 60]. All cells of morphotype *E* are positive with all cytokeratin antibody-preparations examined, including the monoclonal antibody K_G 8.13 (Fig. 6c). This finding is basically in agreement with reports of Cremer et al. [9], Virtanen et al. [69] and Chen [7]. In contrast to colonies of morphotype *ED*, all cells of morphotype *E* have revealed positive reaction with vimentin antibodies (see below) [9]. These cells are negative for desmin.

Colonies of morphotype *AF* [for definition see 35] have been very frequent in some cultures but have represented

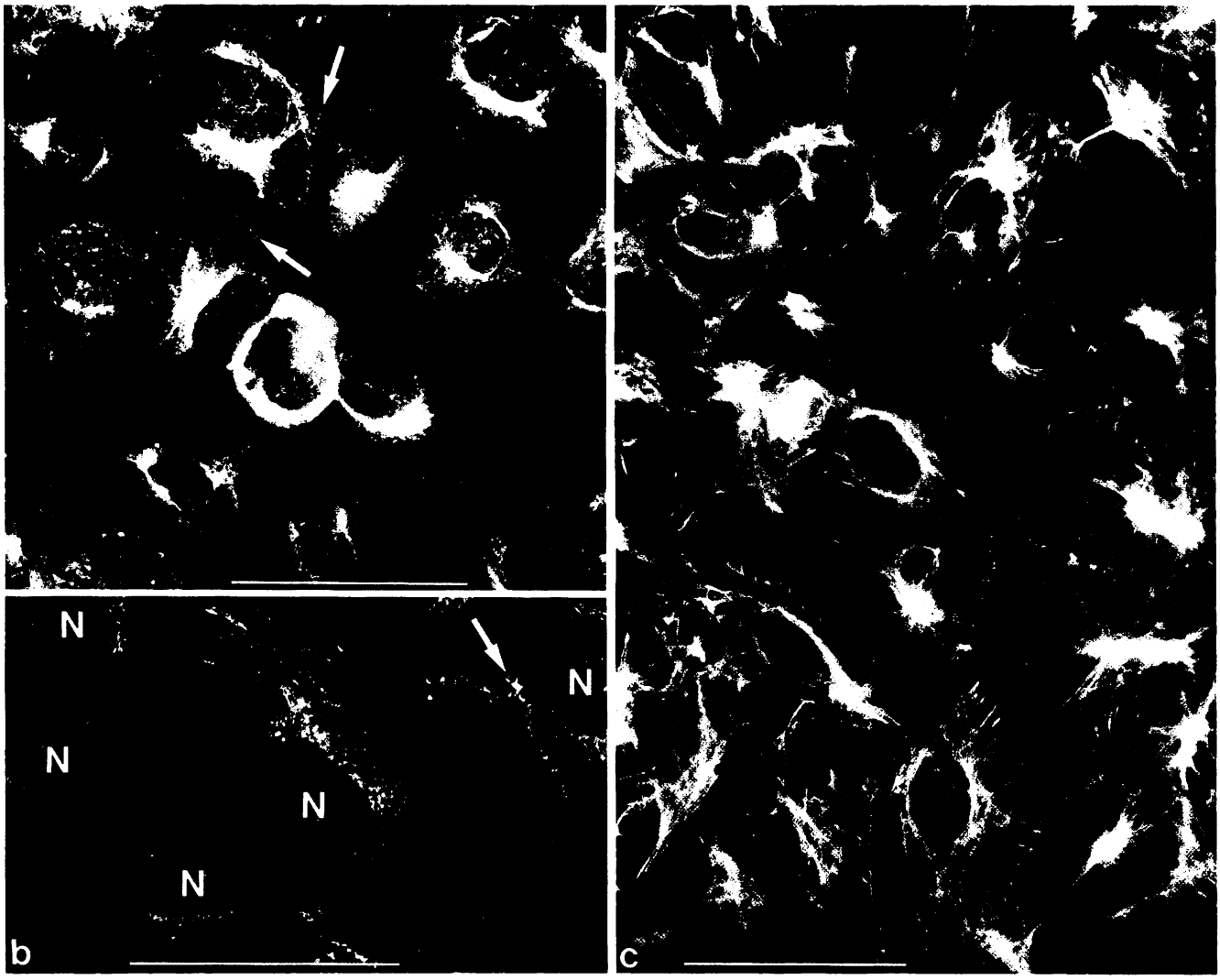

Fig. 6a–c. Immunofluorescence microscopy of amniotic fluid cell cultures of morphotype E after reaction with antibodies to desmoplakin **a, b** and with the monoclonal antibody K_G 8.13 **c**. Note the presence of desmosomes in some (*arrows* in **a** and **b**) but not all cells. These cells show cytokeratin fibril arrays extending throughout the whole cytoplasm, often terminating at distinct sites of cell-to-cell boundaries **c** which by double fluorescence microscopy (not shown here; cf. 52) can be shown to correspond to desmosomes. N, nuclei. *Bars* denote 50 μm

only a minor proportion of cell colonies in other samples grown under identical conditions. Cells of these colonies are rather pleomorphic: in some areas they may still reveal regions of epithelial-like intercellular boundaries, whereas in other areas of the same colony they display a rather fibroblastoidal morphology and form only loose and sparse contacts (e.g., Fig. 7a–d). Correspondingly, colonies of this morphotype also display differences of immunofluorescent reaction within the same colony. While some groups of cells still reveal positive desmoplakin 'dot' staining at cell-to-cell boundaries (Fig. 7a) other cells do not show the typical punctate staining with these antibodies. In addition, some *AF* cells exhibit positive desmoplakin staining on certain cytoplasmic structures, including juxtanuclear 'clouds' of finely punctate desmoplakin material (Fig. 7a) and bizarre-shaped large cytoplasmic aggregates (Fig. 7b; for electron microscopy see below). All cells of *AF*-colonies show fibrillar staining with the two monoclonal cytokeratin antibodies applied (Fig. 7c, d) as well as with conventional antisera to murine liver cytokeratin D (data not shown) [52]. By contrast, antisera raised against bovine epidermal prekeratins have revealed a positive reaction in only a few cells (not shown here; see Fig. 7 of Ref. 9) but not in others, indicating either a difference of expression of cytokeratin polypeptides or a difference of conformation of cytokeratin polypeptides within these filaments. All *AF*-cells have been positive for vimentin (see below and Ref. 9) but negative for desmin (data not shown).

Colonies of morphotype F are made up of cells of an elongated, frequently fusiform, 'fibroblastoidal' morphology and have been repeatedly regarded in the literature as derived from fibroblasts or other mesenchymal cells [41, 69, for review see 35]. Detailed immunocytochemical characterization, however, has shown that these cells are not fibroblasts or smooth muscle-derived cells but represent a special morphotype of epithelial cells highly deviated during growth in vitro. In most cells, staining of these colonies with antibodies to desmoplakin (Fig. 8a) has not shown desmosomal patterns at cell-to-cell boundaries. However, one frequently sees positive punctate fluorescence through-

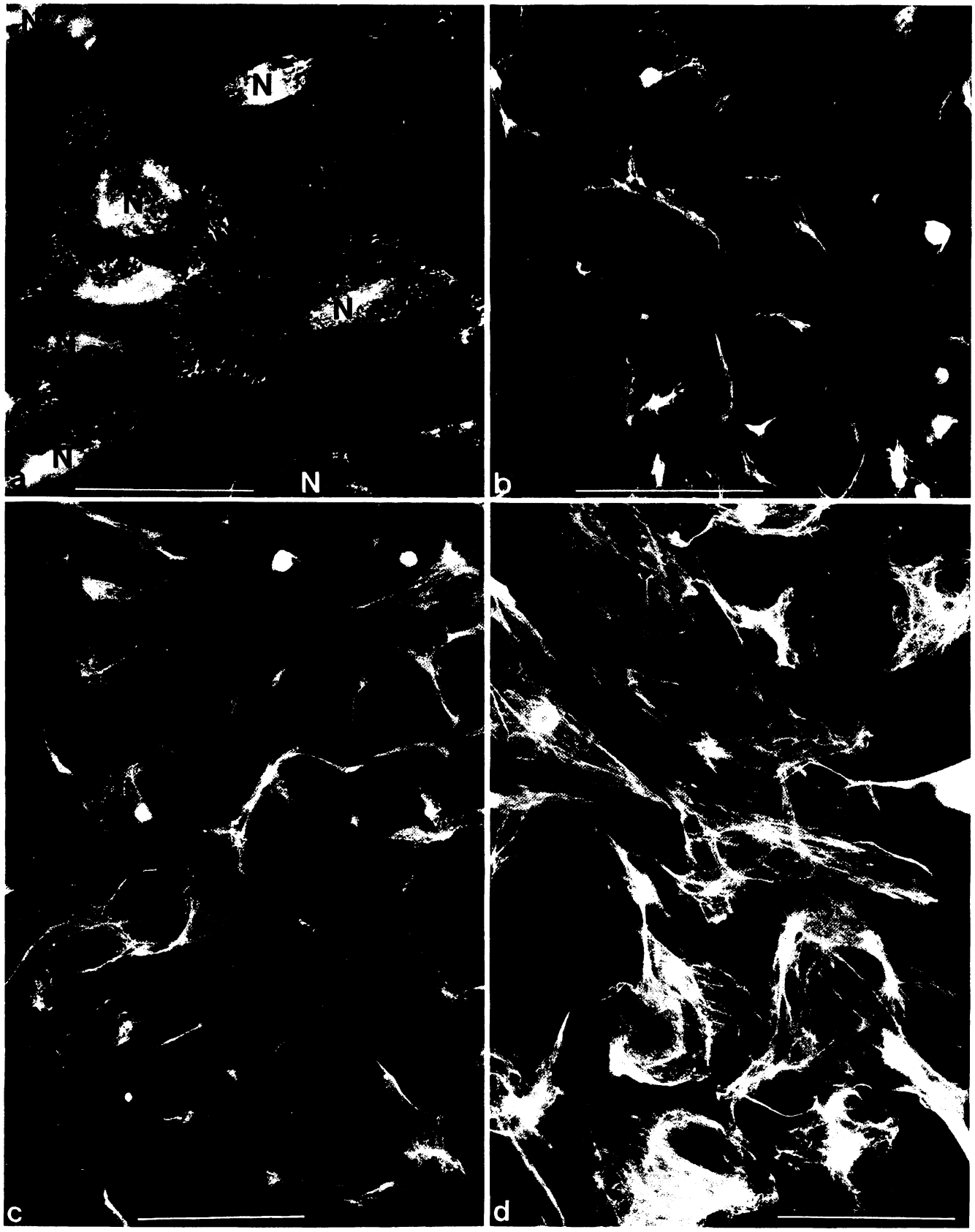

Fig. 7a–d. Immunofluorescence microscopy of cell colonies of AF morphotype stained with different antibodies **a, b.** Reaction with antibodies to desmoplakin. Note differences in staining between different cells, revealing either dotted desmosomal reaction sites at cell-to-cell boundaries or accumulation of desmoplakin material in juxtanuclear cytoplasmic regions (**a**: N, nuclei), or in association with certain cytoplasmic aggregates and fibers **b. c** Reaction with monoclonal antibody K_G 8.13. **d** Reaction with another monoclonal antibody. *Bars* denote 50 μm

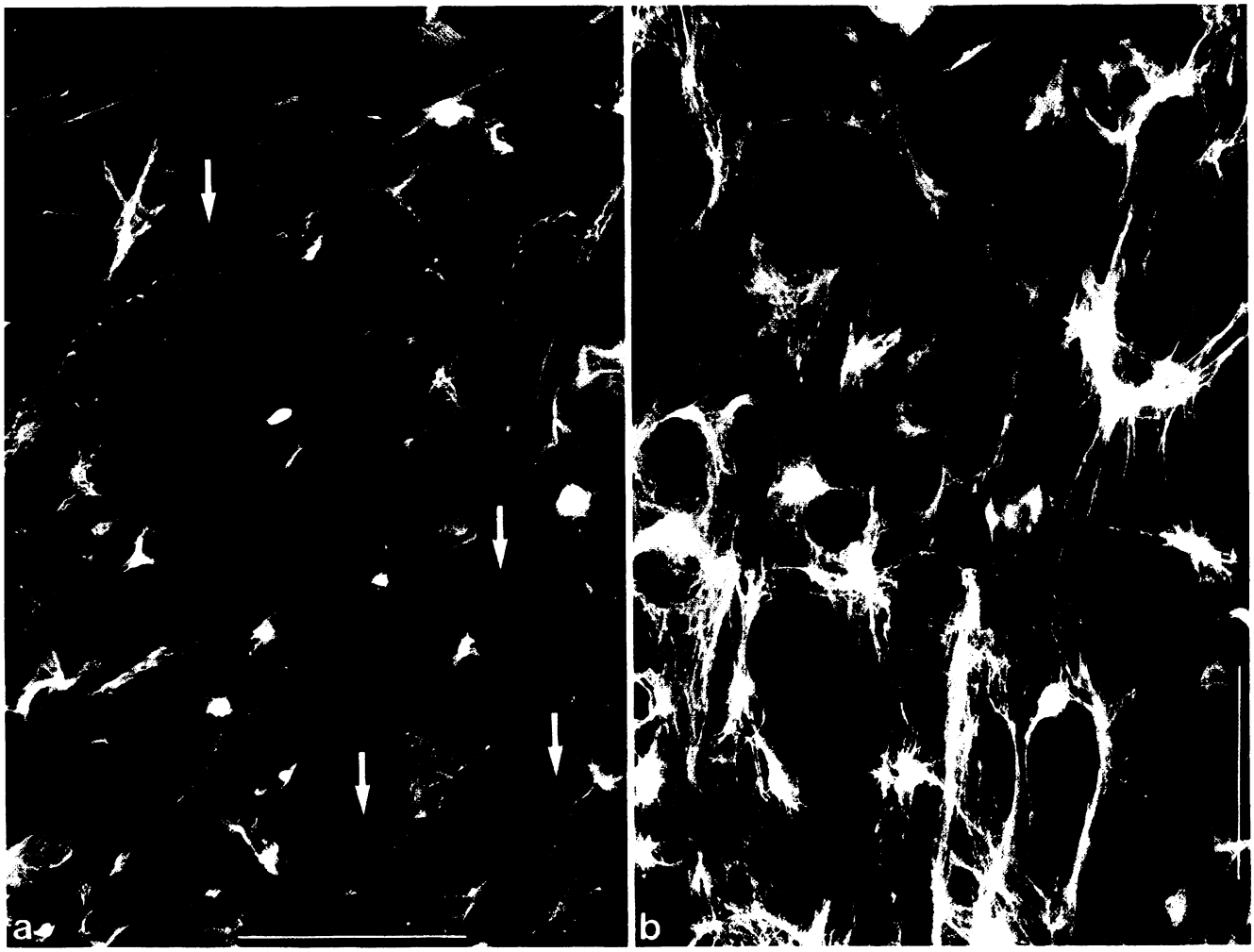

Fig. 8a, b. Immunofluorescence microscopy of cells of colonies of morphotype F derived from amniotic fluids after reaction with antibodies to desmoplakin **a** and monoclonal antibody K_G 8.13 **b**. Note the absence of the typical desmoplakin staining at most cell-to-cell boundaries and the presence of large fluorescent perinuclear aggregates **a** as well as small intracellular dots, probably mostly representing internalized desmoplakin material (*arrows* in **a**). Cytokeratin fibril arrays extend throughout the whole cytoplasm, usually in irregular arrays unrelated to focal membrane sites **b**. *Bars* denote 50 μm

out the cytoplasm (*arrows* in Fig. 8a) as well as on fibrillar structures and certain types of juxtanuclear aggregates of variable sizes, ranging from about 0.5 to 5 μm in diameter (Fig. 8a). This type of desmoplakin distribution has not been encountered so far in any of the numerous permanent cell lines examined [cf. 23, 25, 26, 28, 51, 58] but may reflect endocytotic uptake and probably digestion of desmosomal material (for electron microscopy see below). While cells of *F*-colonies have been negative with some of the antisera against epidermal prekeratin (data not shown; cf. Ref. 8) they have shown extensive fibrillar staining with antibodies to hepatic cytokeratin D [52] and the two monoclonal cytokeratin antibodies available (Fig. 8b). Cells of morphotype *F* invariably are positive for vimentin (see below), whereas no significant reaction has been obtained with antibodies to desmin.

Appearance of vimentin filaments in cell cultures from amniotic fluids

The appearance of intermediate-sized filaments of the vimentin type during culturing of epithelial cells in vitro has been described in both primary cultures and permanent cell lines [e.g., 12, 14, 15, 17, 18, 24, 26, 66, 68]. However, this de novo appearance of vimentin filaments in cultured epithelial cells is not an inevitable consequence of adaptation to culture conditions as has been demonstrated by the absence of vimentin in certain epithelial cell lines, including rat hepatoma MH_1C_1 [24], bovine mammary gland line BMGE + H [58], and the human carcinoma cell lines A-431, MCF-7, and HT-29 [49]. We have therefore examined the appearance and distribution of vimentin-positive cells in cell cultures from amniotic fluids in greater detail. Colonies of such cell cultures have been examined between days 9 and 15 of culture, and identical results have been obtained for all these days. The only type of colony in which vimentin-negative cells predominate is morphotype *ED* (Figs. 9 and 10). However, in some *ED*-colonies, which are generally assumed to be of clonal origin, as has also been discussed for the other morphotypes [35], we have noted individual cells – or clusters of cells – which are faintly but significantly stained with antibodies to vimentin (Fig. 9a–f). In most of these cells such vimentin-positive staining is indistinct (Fig. 9b, c), but characteristic vimentin fibril arrays have also been detected in individual cells (Fig. 9d–f).

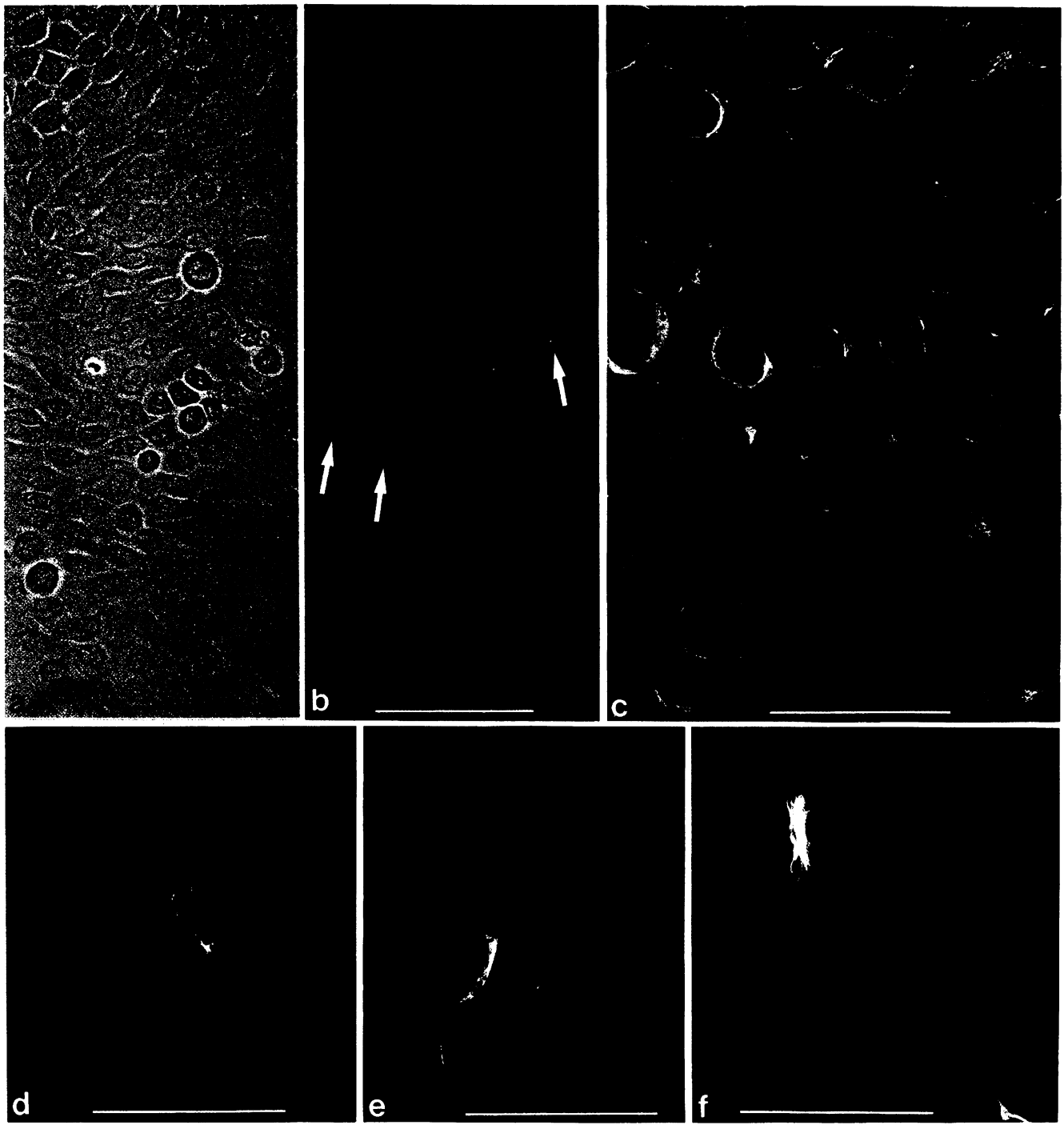

Fig. 9a–f. Light microscopy showing phase contrast **a** and epifluorescence **b–f** optics of amniotic fluid cell culture colonies of morphotype ED after reaction with antibodies against vimentin. **a–c** Note negative reaction in most cells but small sites of positive indistinct fluorescence in a few individual cells (*arrows* in **b, c**). **d–f** Some individual cells in the same colony begin to reveal vimentin fibrils. *Bars* denote 100 μm **a, b** and 50 μm **c–f**

An absence of vimentin filaments has also been observed in *ED*-colonies growing within larger colonies of morphotype *E* which constitutively contains vimentin (Figs. 10 and 11). This shows that the induction of the synthesis of vimentin in cultured epithelial cells is dependent not only on the environmental conditions in the culture dish but also on intrinsic factors present in some cell types (*E*) but not in others (*ED*). Detailed inspection has further revealed the appearance of individual vimentin-positive *ED*-cells within clusters of vimentin-negative *ED*-cells (Fig. 11), and we have even found situations where, in distinct groups of a few cells, probably sister-cells, one cell is vimentin-positive whereas the adjacent cells are negative (Fig. 11a–c). This indicates that even in the largely vimentin-negative *ED*-colonies individual cells can express vimentin filaments.

Although all the other three major morphotypes of cell cultures from amniotic fluids (*E, AF, F*) are positive for both cytokeratin and vimentin, the display of the vimentin

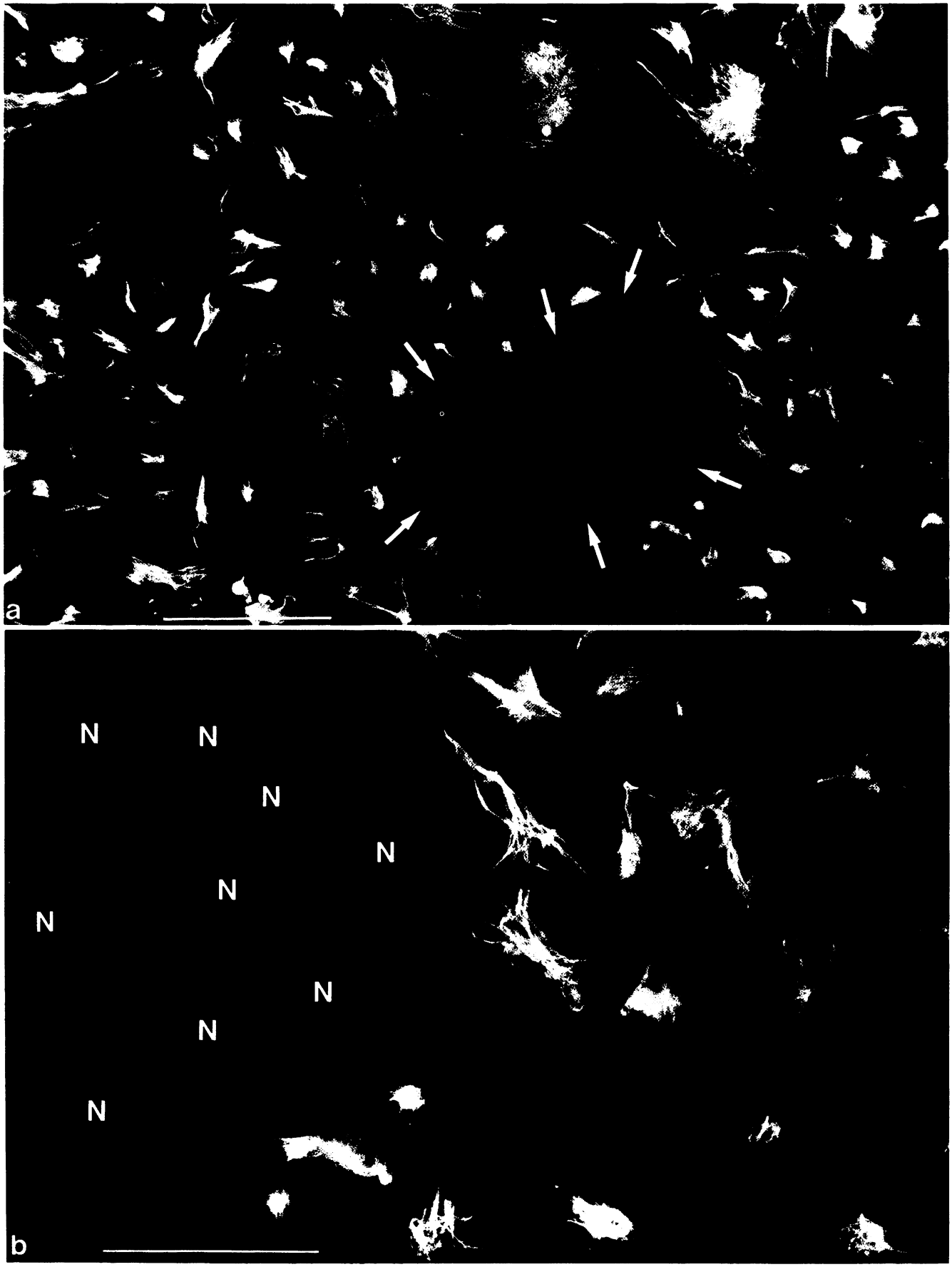

Fig. 10a, b. Immunofluorescence microscopy of cell culture colonies from amniotic fluids after reaction with antibodies to vimentin. Here we present a small colony of morphotype ED (demarcated by *arrows* in **a**) surrounded by cells of a morphotype E colony. Note the selective negative staining of most cells of the ED-colony **a** which are shown at higher magnification (**b**; N, nuclei). *Bars* denote 100 μm **a** and 50 μm **b**

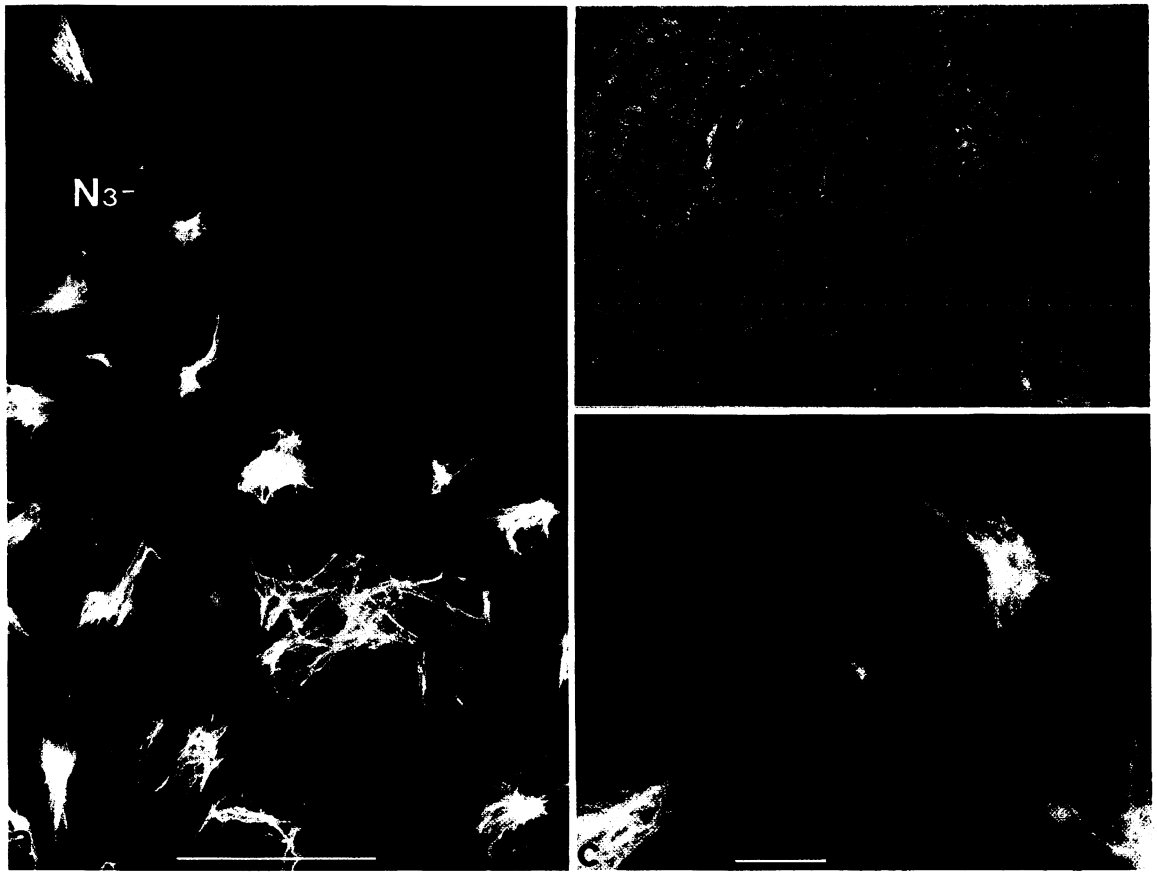

Fig. 11 a–c. Immunofluorescence microscopy of amniotic fluid cell cultures reacting with antibodies to vimentin. **a** Negative colony of morphotype ED compared with positive colony of E-morphotype. Note vimentin production in all cells of morphotype E. **b** Phase contrast and **c** immunofluorescence microscopy of the same group of cells indicated by 'N3' in **a**. Nuclei of this group of cells are numbered N1–N4; note appearance of vimentin fibrils in cells N3 and N4 but not cells N1 and N2 although N1 and N2 are close to N3. *Bars* denote 50 μm **a** and 10 μm **b, c**

filaments is different in the various morphotypes (a survey is presented in Fig. 12a–d). In *E*-colonies the amount and distribution of vimentin filaments can vary from cells containing only small aggregates of vimentin filaments (Fig. 12a) to cells in which vimentin filaments are abundant and spread over most of the cytoplasm (Fig. 12b). In *AF*-colonies, vimentin filaments are abundant and are arranged in irregularly-shaped bundles (Fig. 12c). In *F*-cells vimentin filament bundles are oriented preferentially with the longer axis of the cells and frequently seem to emerge from a conspicuous large and dense juxtanuclear aggregate (Fig. 12d) which also stains positively with the cytokeratin and desmoplakin antibodies used as shown by double immunofluorescence (data not shown; for electron microscopy see below).

Double immunofluorescence microscopy

Comparisons of the specific fibrillar arrays seen after staining with antibodies to cytokeratins and to vimentin have shown that in the few cells of *ED*-colonies in which the two types of intermediate filament proteins simultaneously occur, they do not coincide (data not shown). For example, cytokeratin filaments can usually be seen attached to desmosomes, whereas vimentin filaments seem to be excluded from such 'tonofibrils' (compare Fig. 5 with Figs. 9–11), as has also been reported for other epithelial cell cultures [e.g., 15, 17, 24, 26, 58]. In the colonies of the other morphotypes, however, this distinct and mutually exclusive staining pattern is not always obvious, and frequently fibrillar strands are seen which, by double immunofluorescence microscopy, are positive with both antibodies to cytokeratin and antibodies to vimentin. Careful examination, however, reveals, besides 'double-stained' fibrils, certain fibrils which contain only cytokeratins, including typical desmosome-attached tonofilament bundles (Fig. 13a, b). This illustrates the specificity of the two types of antibodies used as well as the presence of two different types of filament bundles in these cells, i.e., pure cytokeratin bundles and others which contain filament subunit proteins of both types, cytokeratin and vimentin. At the level of the light microscopic resolution, however, we cannot decide whether in the latter type of bundles the vimentin and the cytokeratin molecules are located in different filaments.

Electron microscopy

Cell colonies of all morphotypes have been fixed as grown in monolayer cultures and examined by electron microscopy. Colonies of morphotypes *ED*, *E*, and *AF* display normal structures also known to occur in many other cultured epithelial cells. Of special interest has been the identification of the type of junctions at the intercellular boundaries of *AF* and *F* colonies. Typical desmosomes have not been recognized in *F* cells, in agreement with our immunofluores-

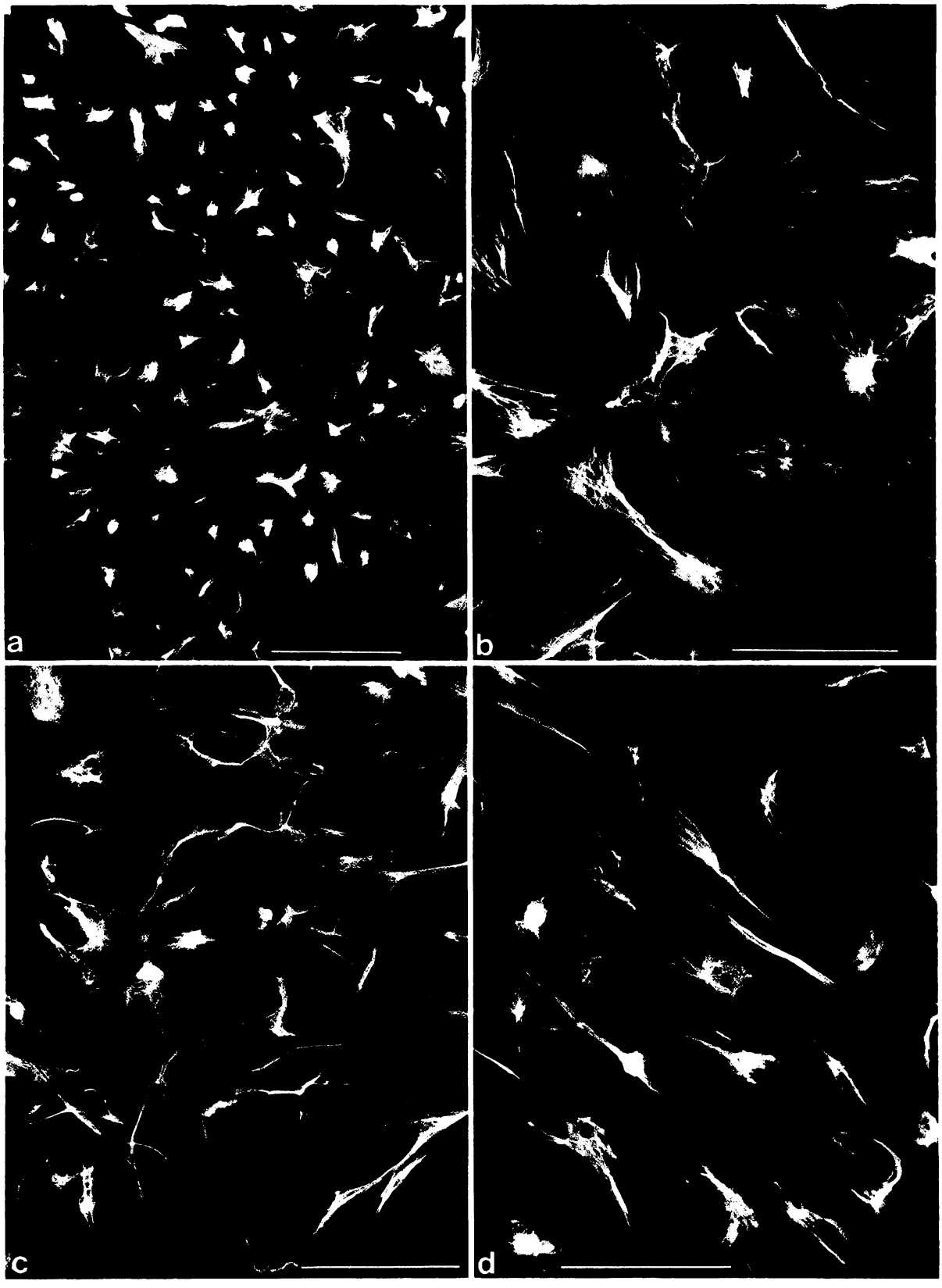

Fig. 12a–d. Comparison of immunofluorescence microscopy of various morphotypes of cell colonies grown in amniotic fluid cell cultures, after reaction with vimentin antibodies. **a, b** Morphotype E; **c** morphotype AF; **d** morphotype F. *Bars* denote 100 μm **a** and 50 μm **b–d**

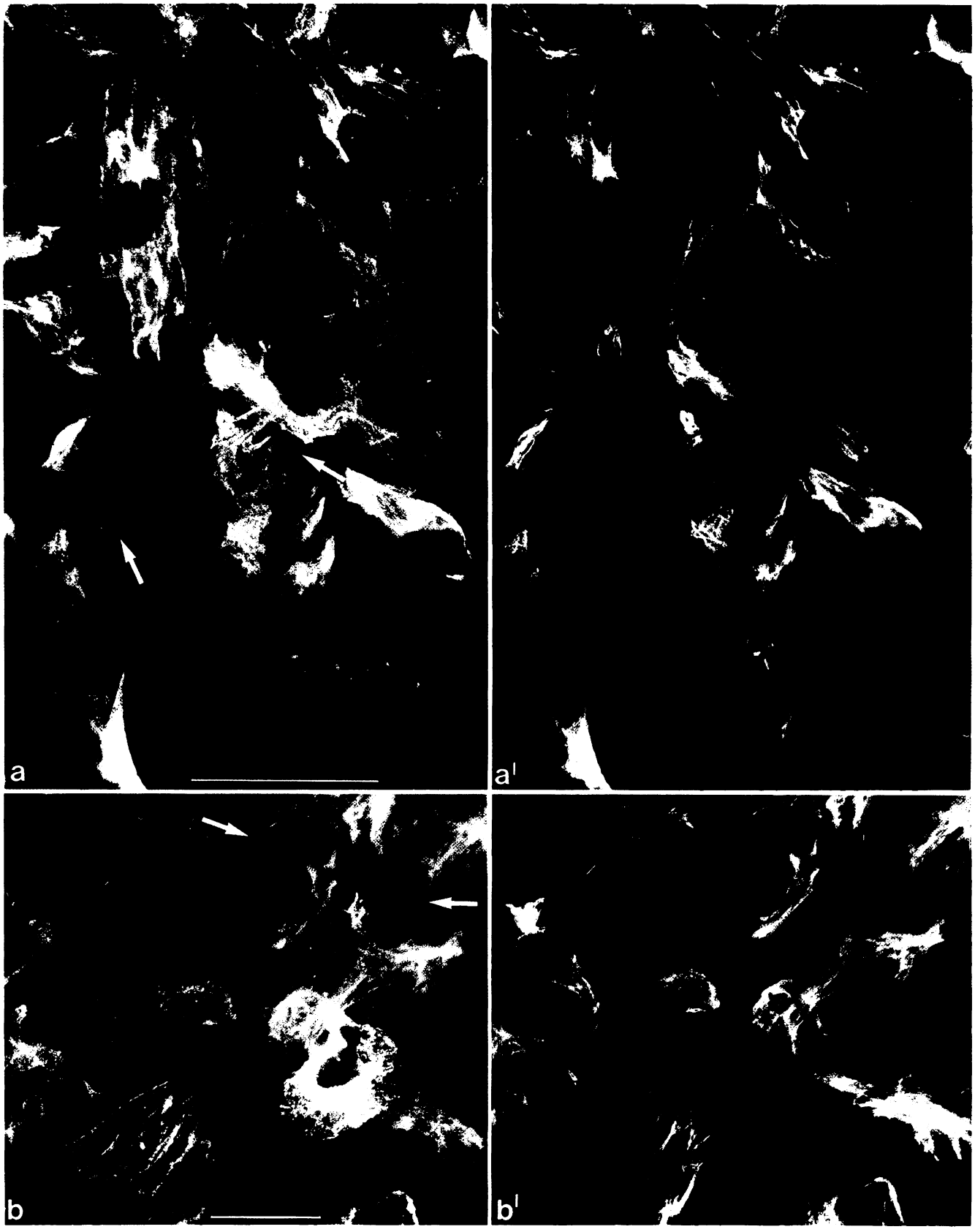

Fig. 13a–b. Double label immunofluorescence microscopy of cell colony of morphotype E treated with murine monoclonal cytokeratin antibody K_G 8.13 **a, b** and guinea pig antibodies to vimentin **a′, b′**. Note that the fibril staining coincides in many regions but also that differences of fibrillar decoration with antibodies can occur (some regions showing fibrils positive with the cytokeratin antibody only are denoted by *arrows*). *Bars* represent 50 μm **a, a′** and 25 μm **b, b′**

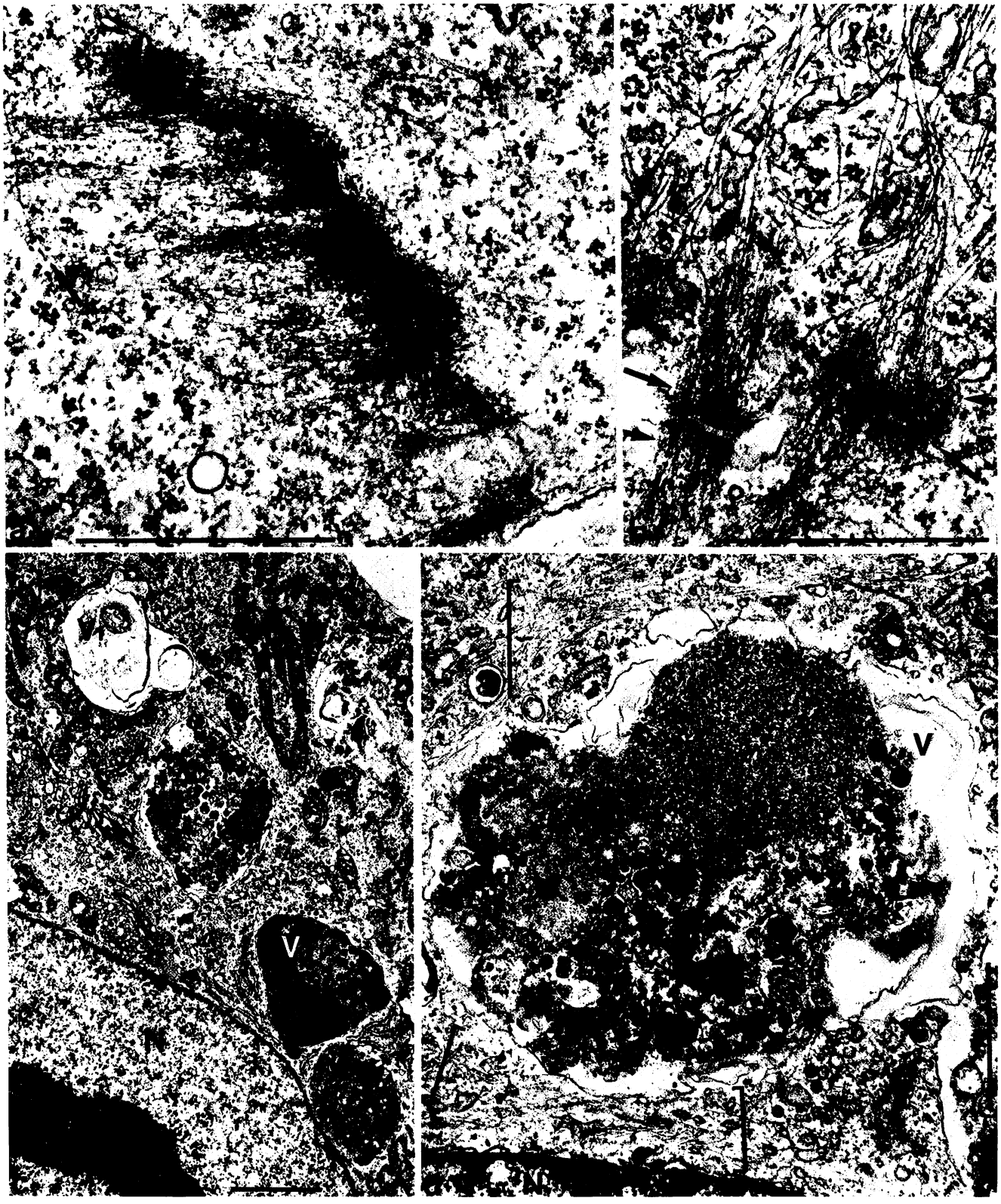

Fig. 14a–d. Electron micrographs of cultured cells from human amniotic fluids (morphotype F) as revealed in these sections parallel to the substratum. Cell-to-cell boundaries contain special junctions (*arrows* in **b**) which are characterized by an intramembranous space of about 20–30 nm, sometimes revealing a midline-like structure, and are associated on either cytoplasmic side with an indistinct fuzzy coat of electron-dense material at which 5–6 nm microfilaments attach **a, b** whereas intermediate-sized filaments (one is denoted by an *arrow* in **a**; they are abundant in the upper half of **b**) do not attach at these junctions. These cells contain large juxtanuclear vacuoles (designated V1–V4 in **c** and V in **d**) which are filled with various materials. Note that these vacuoles are closely surrounded by fleeces of intermediate-sized filaments (denoted by brackets in **d**). N, nucleus. *Bars* denote 1 μm **a, b, d** and 2 μm **c**

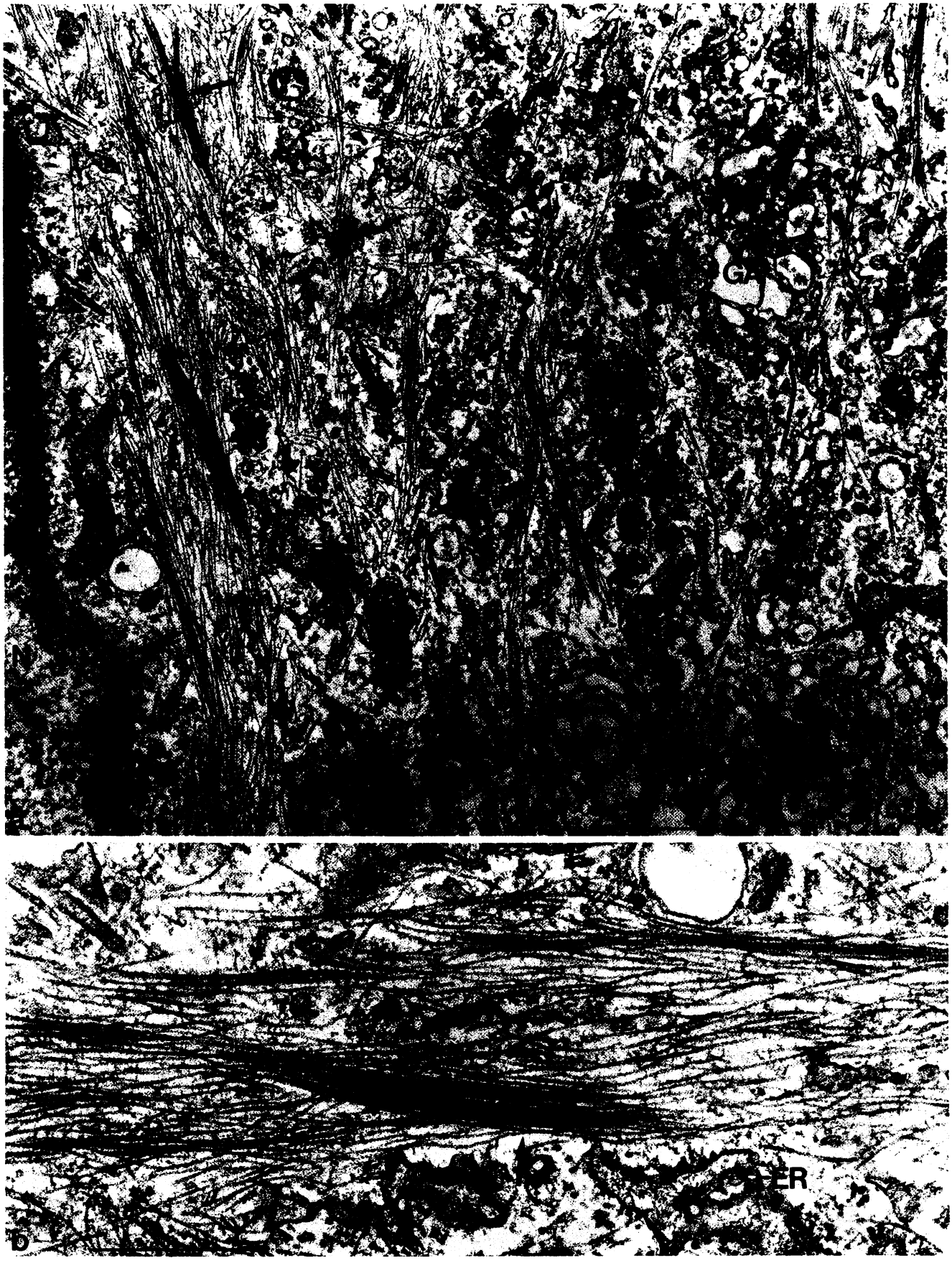

Fig. 15a, b. Electron microscopy (similar to Fig. 15) of cultured cells from human amniotic fluid, showing the abundance of intermediate-sized filaments in cells of colonies of morphotype F. **a** Survey micrograph of juxtanuclear cytoplasm (N, nucleus; ER, rough endoplasmic reticulum; GA, Golgi apparatus) showing the occurrence of both loosely arranged fleeces and densely fasciated bundles (some are denoted by *arrows* in **a**) of intermediate-sized filaments. **b** Details of intermediate filament organization in loose fleeces and in tightly packed, densely stained bundles closely reminiscent of typical cytokeratin tonofibrils (e.g., *arrow*). *Bars* denote 1 μm **a** and 0.5 μm **b**

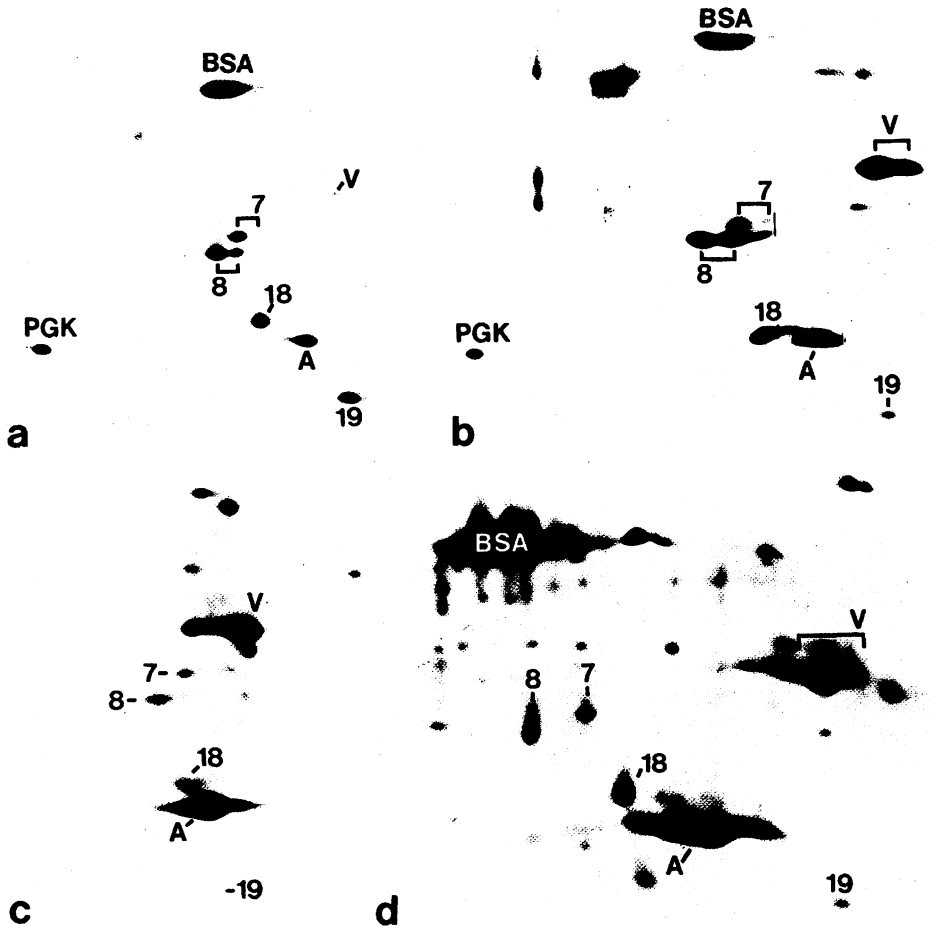

Fig. 16a–d. Two-dimensional gel electrophoresis of cytoskeletal polypeptides from various morphotype colonies of cultured human amniotic fluid cells (**a–c**, non-equilibrium pH gradient electrophoresis in first dimension; **d**, isoelectric focusing in first dimension; other conditions as described in Ref. 49). **a** ED-colony; **b** E-colony; **c** and **d** F-colony. Cytokeratin polypeptides are designated by Arabic numerals as described by Moll et al. [49]. V, endogenous vimentin. BSA, bovine serum albumin added as reference; A, rabbit muscle α-actin added as reference. Note that only cytokeratins Nos. 7, 8, 18, and 19 are identified and that more basic cytokeratin polypeptides appear to be missing

cent findings of an absence of desmoplakin-positive dots at the cell-to-cell boundaries. Frequently, we have observed clusters of cell junctions with a finely dotted 'midline structure' and a fuzzy, densely stained, cytoplasmic coat at which not intermediate-sized filaments but microfilament bundles attach (Fig. 14a, b). This type of junction resembles desmosomes in some structural aspects but it is different from true desmosomes in both the organization of the cytoplasmic plaque and the absence of associated bundles of intermediate-sized filaments. A similar type of junction has been described in other cultured epithelial cells [73] and in cultured eye lens-forming cells [54].

Cells of colonies of morphotype *F*, and to some extent also *AF* cells often contain, in the juxtanuclear cytoplasm, one or several vacuoles which vary in size and content (Fig. 14c). Such vacuoles could attain diameters of up to 5 μm (Fig. 14d). Some of them appear relatively electron-translucent, some contain membrane-like myelin whorls, and some are filled with various cellular materials and are reminiscent of autophagic vacuoles (Fig. 14c, d). These juxtanuclear vacuoles, which characteristically are surrounded by masses of intermediate-sized filaments (Fig. 14d), have been shown by immunoelectron microscopy to be identical to the dense granules positively stained with antibodies to a variety of cytoskeletal proteins (cf. Figs. 7 and 8). They resemble the juxtanuclear formations described in rat hepatoma cells of line 72/22 [5]. The typically high density of intermediate-sized filaments in *AF*- and *F*-cells is illustrated in Fig. 15a. Besides relatively loose, frequently irregular arrays of intermediate filaments which predominate in most cells, regions of densely fasciated intermediate-sized filaments, reminiscent of typical tonofilament bundles of epithelial cells [13, 17, 26], are also recognized.

Gel electrophoresis

We have prepared, by extraction with high salt buffers and Triton X-100, cytoskeletal residues from cell colonies of all four morphotypes and have analyzed their polypeptide patterns by two-dimensional gel electrophoresis, using non-equilibrium pH gradient electrophoresis or isoelectric focusing in the first dimension analysis. In most cases, the separated polypeptides have been visualized by silver staining. Cell colonies of all morphotypes contain cytokeratin polypeptides Nos. 7, 8, 18, and 19 (for designations see Ref. 50), the latter being present in variable amounts (Fig. 16a–d). Vimentin has been recognized in *ED*-cells only in miniscule amounts, whereas it represents a major cytoskeletal protein in the colonies of all other morphotypes. Cytokeratin polypeptides more basic than polypeptide No. 8 have not been found.

Discussion

At the time when amniocentesis is usually performed, the human fetus has already developed typical epithelial tissues expressing two of the most prominent epithelial markers, i.e., cytokeratins and desmoplakins [this study and 50]. In

addition to many dead and non-proliferative cells, amniotic fluids contain certain cells which can adhere to an artificial substratum, grow in monolayer, and proliferate. Our observations are in agreement with previous reports of the occurrence of morphologically different cell types in such cultures [1, 8, 9, 29, 33, 35, 41, 69, 70]. Such morphologic differences have mostly been interpreted as due to origins of these cell colonies from different cell types of the fetus. The finding that practically all the different morphotypes of cell colonies grown from cells suspended in the amniotic fluid contain intermediate-sized filaments stained with cytokeratin antibodies and, on gel electrophoresis, reveal cytokeratin polypeptides as major components of their cytoskeletons, supports the notion that all major cell types growing in such cultures are of epithelial character and origin [cf. 7, 9, 69]. Cells completely negative for cytokeratins but positively stained with vimentin, i.e., putative mesenchymally derived cells, are extremely rare in such cultures [69] and are unlikely to contribute to cell cultures as they are used for prenatal diagnosis. We have also not found significant amounts of desmin in any of these amniotic fluid cell cultures and we interpret the polypeptide tentatively designated as 'desmin' in some references [35, 41] as erroneously classified; most probably the polypeptide under question in these publications represents vimentin.

Surprisingly, however, the antigenic determinants exposed on the cytokeratin filaments present in the different types of cell colonies reveal marked differences. For example, cells of some of these colony morphotypes are stained with certain antibodies to bovine epidermal prekeratin whereas others do not [this study and 9]. This could reflect a different origin of these colonies from different fetal epithelia [9], as various epithelia can contain different sets of cytokeratin polypeptides [20–22, 26, 49, 71] and are stained differently by various cytokeratin antibody preparations [2, 18, 19, 50, 67]. On the other hand, however, our gel electrophoretic analysis of cytoskeletal proteins has shown the presence of the same cytokeratin polypeptides Nos. 7, 8, 18, and 19 in all these different colony morphotypes. Hence we are left with the remaining alternative possibility that these different types of colony express the same cytokeratins but differ in the arrangement of the individual cytokeratin polypeptides, at least in the accessibility of certain immunologic determinants to specific antibodies. This conclusion is not without precedent. Franke et al. [18] have observed that the cytokeratin filaments present in murine hepatocytes do not react with certain antisera to epidermal prekeratin whereas those present in cultured hepatocytes do, although in both cell states the same major cytokeratin polypeptides ('A' and 'D', equivalent to human cytokeratins No. 8 and 18) are formed. Such examples emphasize the importance of the use of diverse cytokeratin antibodies in determining the epithelial character of a given cell type. They also demonstrate that such antibody reactions cannot generally be interpreted in terms of expression of different cytokeratin polypeptides, as opposed to the alternative explanation of altered arrangements of identical cytokeratin polypeptide chains.

Our results confirm our previous finding [9] that most morphotypes of cell colonies grown from amniotic fluids express vimentin filaments, in addition to cytokeratin filaments, often in similar-looking fibrillar arrays (for related observations in certain heterokaryons see Ref. 45). In fact all morphotypes, with the exception of the majority of cells of *ED*-colonies, express vimentin filaments, similar to many other cultured epithelial cells [12, 14, 15, 17, 18, 24, 26, 58, 66, 68]. In view of the absence of significant vimentin antibody staining in all epithelial tissues examined so far [26, 47, 53; for the dubious case of amnion epithelium see 9], we do not have a plausible explanation for the phenomenon that vimentin appears in large amounts in all colonies of morphotypes *E*, *AF*, and *F*, but not *ED*. However, we think it is unlikely that this is due to the exclusive origin of *E*-, *AF*-, and *F*-cells from a certain subpopulation of cells present in the amniotic fluid which initially contain both vimentin and cytokeratin and have a higher proliferative potential, as suggested by Virtanen and colleagues [42, 68]. Rather we interpret the appearance of vimentin filaments in a few cells of *ED*-colonies as support for our notion [e.g., 15, 17, 24, 25] that vimentin expression is a phenomenon induced during culturing in vitro, under control of both extrinsic and intrinsic factors, as is also suggested by the finding that vimentin expression can start in primary cultures of non-dividing rat hepatocytes [24].

Differences in cytoskeletal organization in different colony morphotypes of amniotic fluid cells cultured in vitro are not restricted to differences in the exposure of certain cytokeratin determinants and the appearance of vimentin in *E*-, *AF*-, and *F*-colonies. Drastic differences are also observed in the display of desmosomes and desmosomal plaque proteins. While *ED*-colonies appear as typical 'normal' epithelial monolayers interconnected with numerous desmosomes, *E*-cells reveal such desmosomes along the boundaries of some cells but not in other cells of the same colony. In addition, *E*- and *AF*-cells show relatively high proportions of internalized, probably vesicular, desmoplakin-rich structures. Endocytotic internalization of desmosome-derived membrane domains is commonly observed in cell cultures transferred by treatment with trypsin and EDTA [23, 43, 60; see there for further references], but it is difficult to understand how such intracellular uptake could occur in a primary culture continuously kept in growth medium. The colonies most deviated from typical epithelial morphology, i.e., morphotypes *AF* and *F*, also show the greatest reduction in ordered arrays of 'dotted' lines seen after desmoplakin antibody staining, in agreement with the electron microscopic finding of a nearly complete absence of typical desmosome in *F*-cells. Thus, *AF*- and *F*-cells present examples of reduction of desmosome formation during a relatively short period of culturing in vitro without experimentally induced cell dissociation and cell detachment.

We also have to consider the possibility that various cell types and forms of cell aggregates may already occur in the initial amniotic fluid. The appearance of individual epithelial cells in such a freely suspended form would not be trivial and would require the detachment of these cells from the underlying tissue as well as the splitting of all intercellular junctions. Presently, the mode of shedding of viable epithelial cells into the amniotic fluid is still unknown. Hence it cannot be excluded that the four major morphotypes of epithelial cells (*ED, E, AF, F*) are related to different stages of cell dissociation rather than to different epithelia.

It would be important to know from which epithelium (or epithelia) the colonies observed after in vitro culture of cells from human amniotic fluid samples are derived. According to their specific cytokeratin pattern, especially

in view of the absence of any basic cytokeratins, an origin from differentiated epidermal cells can be excluded as well as an origin from practically all other stratified squamous epithelia (for tissue- and cell type-specific patterns of cytokeratin polypeptides see Ref. 49). We cannot exclude, however, that these cells, or at least some of them, are desquamated cells of the fetal periderm which appear to contain a more simple cytokeratin polypeptide composition than the underlying epidermal cells (for detailed discussion of literature see Ref. 50). Of some relevance to problems of prenatal diagnosis is the difference of the cytokeratin polypeptide pattern of these cultured amniotic fluid cells from the pattern of human amnion epithelium which includes two basic cytokeratins (polypeptides Nos. 5 and 6) [cf. 49]. This makes it very unlikely that cells grown from amniocentetic samples are derived from the amnion epithelium (for detailed comparison see also S. Regauer, W.W. Franke, R. Molle, M. Cremer and T.Cremer, manuscript in preparation).

Thus, in comparison of the cytokeratin polypeptide patterns described in this study with those present in epithelia of adult human tissues we are left with a few likely candidate tissues. Fetal urine would be a possible source for viable cells present in the amniotic fluid, in particular since it has been shown that cultured cells from neonatal and fetal urine in vitro form colonies similar to *E*- and *AF*-type colonies of amniotic fluid cells [10, 34] and continue to express cytokeratin filaments [11]. In contrast to amniotic fluid cells in culture, however, urothelial cells can contain a certain amount of a basic cytokeratin (component No. 5). However, since we cannot exclude that urothelial cells desquamated into the urine include different cell types, those from the upper layer and basal cells, we cannot definitely exclude a derivation of the in vitro colonies of amniotic fluid cells from a subpopulation of urothelial cells. Other candidates would be certain subpopulations of cells of the respiratory and digestive tract (for comparison of polypeptide patterns see Ref. 49). Clearly, more detailed cell type characterization, also utilizing other cell type-specific markers and fetal tissues for comparison, is necessary before one can decide from which fetal epithelia these cultures have been derived. Whether the different reactivities with certain antibodies to cytokeratins are related to the variable specific amounts of the small cytokeratin No. 19 also remains to be examined.

One of the most important conclusions one should draw from studies such as this is the importance of non-morphologic markers in studies of cells growing in vitro, including immunocytochemical and biochemical criteria. For example, *F*-cells in cultures of cells from amniotic fluids, no matter how much they look like fibroblasts, are still identifiable as epithelium-derived cells by the presence of certain cytokeratins. It is hoped that the increasing number of antibodies against defined cell-type-specific components will be helpful in identifying and characterizing cells growing in vitro, including those grown in cultures of amniotic fluid cells.

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