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Amniocentesis
prenatal diagnosis
tissue culture

Cultivated Cells from Diagnostic Amniocentesis in Second Trimester Pregnancies. I. Clonal Morphology and Growth Potential

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Extract

Amniotic fluid obtained transabdominally for prenatal diagnosis (mean length of amenorrhea 16.1 weeks) contained between 10^3 and 10^5 cells/ml, the great majority of which were squamous; their labeling index with (^{3}H)thymidine was $< 2 \times 10^{-3}$. An average of 3.5 clones ($> 10^2$ cells)/ml fluid grew; of these, an average of 1.5 grew for >20 population doublings (CPD). Of 288 clones analyzed from 20 cases, 271 could be classified into one of three groups: *group I,* 24 were typical fibroblast-like cells (F cells) comparable with control clones from neonatal dermis with macroscopic "ribbing" growth pattern of megaclones, preponderance of spindle-shaped cells growing in parallel arrays, and high growth potential (mean of selected clones = 53 CPD); *group II,* 67 were typical epithelioid types (E cells) consisting of cells with intimate cell-to-cell contact which were resistant to trypsin detachment, with poor subcloning efficiencies and poor growth potential (mean of selected clones 14.5 CPD); *group III,* the predominant class with 180 clones, was of a type not described previously (AF cells); their megaclones had "bulls-eye" growth patterns, individual cells were pleomorphic, and growth potentials were intermediate to those of E and F cells. This tripartite classification holds for the majority of samples, but individual variation is suggested by the observation of exceptional clonal types sharing characteristics of both the E and AF class.

Speculation

The AF cell is the predominant type in most diagnostic amniotic fluid cultures; heretofore, such cultures were widely regarded as consisting primarily of fibroblast and epithelial-like cells. Skin fibroblast cultures are therefore inappropriate controls for the interpretation of constitutive and induced levels of enzymes in such cultures. Established amniotic fluid cell cultures are derived from a single or a few clonable cells with high growth potential and therefore may not always be representative of the fetus.

When fluid is withdrawn from the amniotic sac of nonexceptional (23) midterm pregnancies and placed into culture, a small number of cells proliferate. It is well established that these cells are of fetal origin, although their tissue (or tissues) of origin remain speculative (*e.g.,* 1, 8, 20). Since this heterogeneous cell population is widely used for the prenatal diagnosis of genetic disease, it is important to characterize its clonable constituents to the greatest possible extent. The basal activities of various enzymes have been shown to vary according to the cell type and tissue of origin (4, 9, 19). Perhaps more important for future developments, the success with which enzyme markers may be inducible in such cultures is likely to depend on the constituent cell types. Our approach to this problem was to compare clonal and subclonal growth patterns of amniotic fluid cells with a standard cell type, fibroblast-like cells derived from neonatal skin (dermis) (14). We found the major clonable cell type derived from amniotic fluid was quite distinct, and so far as we could ascertain, had not previously been considered separately from fibroblast-like cells.

MATERIALS AND METHODS

Amniotic fluid was obtained from 32 pregnancies monitored for genetic disease. From each tap generally 15–20 ml were obtained, of which at least 12 ml were used for the respective cytogenetic or biochemical diagnosis and the remainder for the experimental protocols. Taps which yielded less than 15 ml were not used for these studies. The mean time of amniocentesis was 16.1 weeks (range: 14–24 weeks) from the first day of the last menstrual period. Cultures were prepared within 2 hr after the tap. General culture methods and media (Dulbecco-Vogt with 16% heat-inactivated fetal calf serum and 100 units/ml penicillin) have been described (5, 17). Plastic ware was used throughout (27).

PREPARATION OF PRIMARY AND SECONDARY CLONES AND ASSAYS OF REPLICATIVE LIFE SPANS

Twenty samples of 3–5 ml uncentrifuged fluid were each mixed with 80 ml complete media; 2.5-ml aliquots of this mixture were dispensed into a series of 35-mm petri dishes containing 22 mm^2 coverslips. There was a complete change of media at *day 5* or *6* and every 2–3 days thereafter. Screening for growth started at *day 10* or *11* all clonal foci of at least 20–50 cells were scored and transferred to a 25 cm^2 culture flask within 4 weeks after initiation, or as soon as they reached a diameter which exceeded 2–3 mm. The initially high dilution of the amniotic fluid samples resulted in no more than 10–20% of the dishes with multiple colonies. In such instances, isolation was attempted by breakage of the coverslip, or by selective trypsinization, if one of the colonies had attached to the area outside the coverslip. The cells were allowed to grow in the 25-cm^2 flask for a maximum period of 2 weeks, at which point they were subjected to further trypsinization and to each of the following procedures: *group a,* hemocytometer counts; *group b,* subcloning by dilute

plating of 100 cells/ml into 60-mm petri dishes; *group c,* continuous *in vitro* passage of standard innocula of 5×10^4 cells/75 cm^2 flask; *group d,* plating of all remaining cells for chromosome preparations, which were harvested 48–72 hr later. The subcloning dishes received one feeding at *day 9* after plating and were stained at *day 12–14* as described below for the megaclones. *Group c* cells intended for continuous *in vitro* propagation received two weekly feedings and were passaged at 14-day intervals or when confluent. They were terminated when counts were less than or equal to the innoculum at the end of the 2 week period. Determination of the *in vitro* life-spans was by estimation of the cumulative population doublings (17). Surplus cells from the *group c* cultures were used for cytographic analysis (28); they were negative for mycoplasma (11) (courtesy of G. Kenny).

PREPARATION OF MEGACLONES

Six to 9 ml of uncentrifuged fluid from nine cases were mixed with 80–120 ml complete media and plated (in 15-ml portions) on glass slides (2 by 3 inches and 1 by 3 inches) in square phage dishes (29). These dishes were incubated for 20 days, with intermittent feedings at 4-day intervals, starting on *day 6.* Staining was with 1% crystal violet in 20% ethanol. Clonal dimensions and cell counts were determined on projections magnified 55 times (30).

RESULTS

When 8-ml aliquots from three separate samples were incubated for 24 hr with equal amounts of media and 2 μCi/ml tritiated thymidine and autoradiographed (31), no labeled nuclei were found among 2×10^3 cells examined. These nonreplicating cells exhibited rather uniform squamous morphologies and only rarely were observed to attach to the culture vessels. Among the few cells which did attach, a large proportion consisted of irregular, bizarre types which failed to proliferate even after prolonged periods of incubation (Figs. 1 and 2). Developing colonies were initially heterogeneous except for epithelial-type cells, which were often in sheets; many of the early clonal foci were "abortive" and frequently produced acid mucopolysaccharides (12) (Fig. 2, *e* and *f*).

PRIMARY CLONES AND THEIR DERIVATIVE MASS CULTURES

Of the 10^3–10^5 cells/ml amniotic fluid, only an average of 3.45 (range: 0.75–7.3) gave rise to detectable colonies (Table 1). Less than half of these achieved a population equivalent to 20 or more CPD, perhaps a minimum requirement for many biochemical assays. A total of 288 primary clones were observed for 82 ml amniotic fluid derived from 20 different donors (Table 1). In comparing the morphologies of the living cultures to our standard of neonatal dermal fibroblasts, it

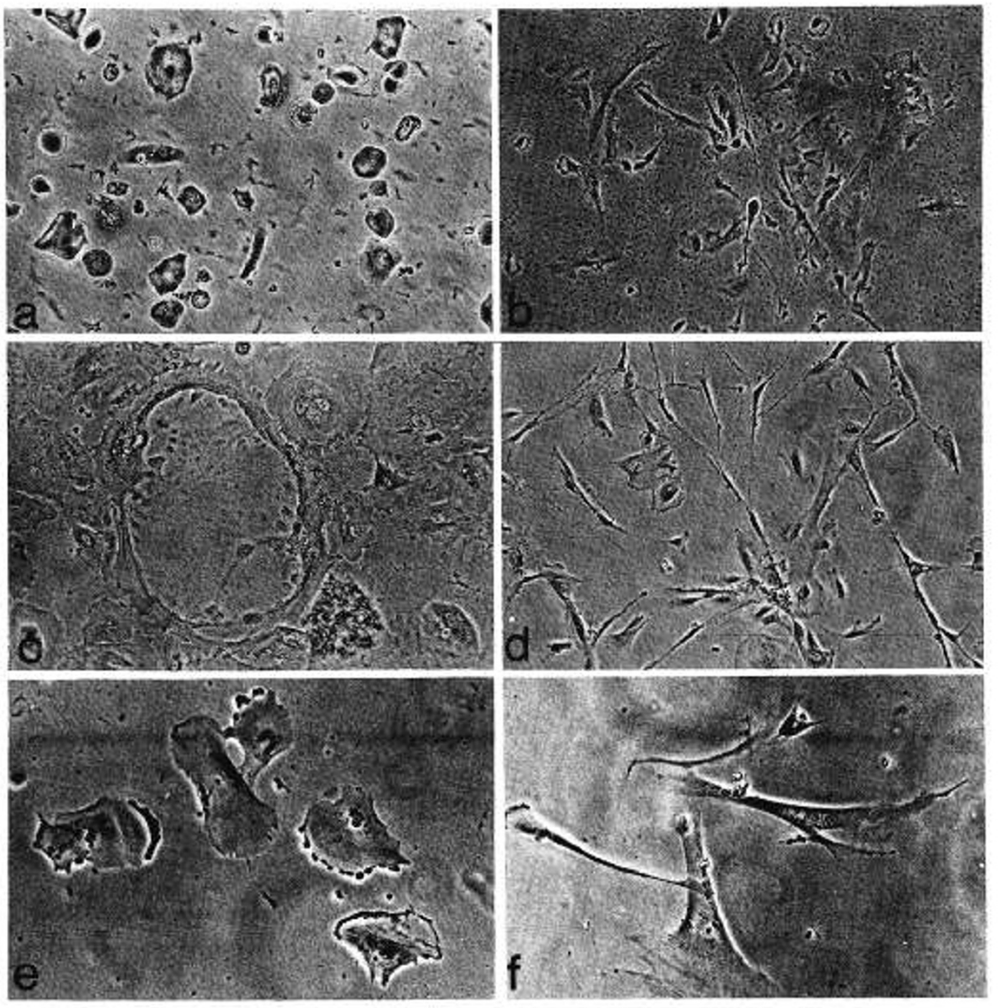

Fig. 1. Morphology of amniotic fluid cells before organized clonal growth (*days 2–6*). *a:* floating squamous cells 48 hr after initiation, two slightly elongated cells in the process of attaching; *b–d:* heterogeneity of early clonal growth; *e–f:* coexistence, at early stages of clonal growth, of epithelioid type and fibroblast-like morphologies (magnified from a single colony). *a–d:* phase contrast, × 63; *e–f;* × 126.

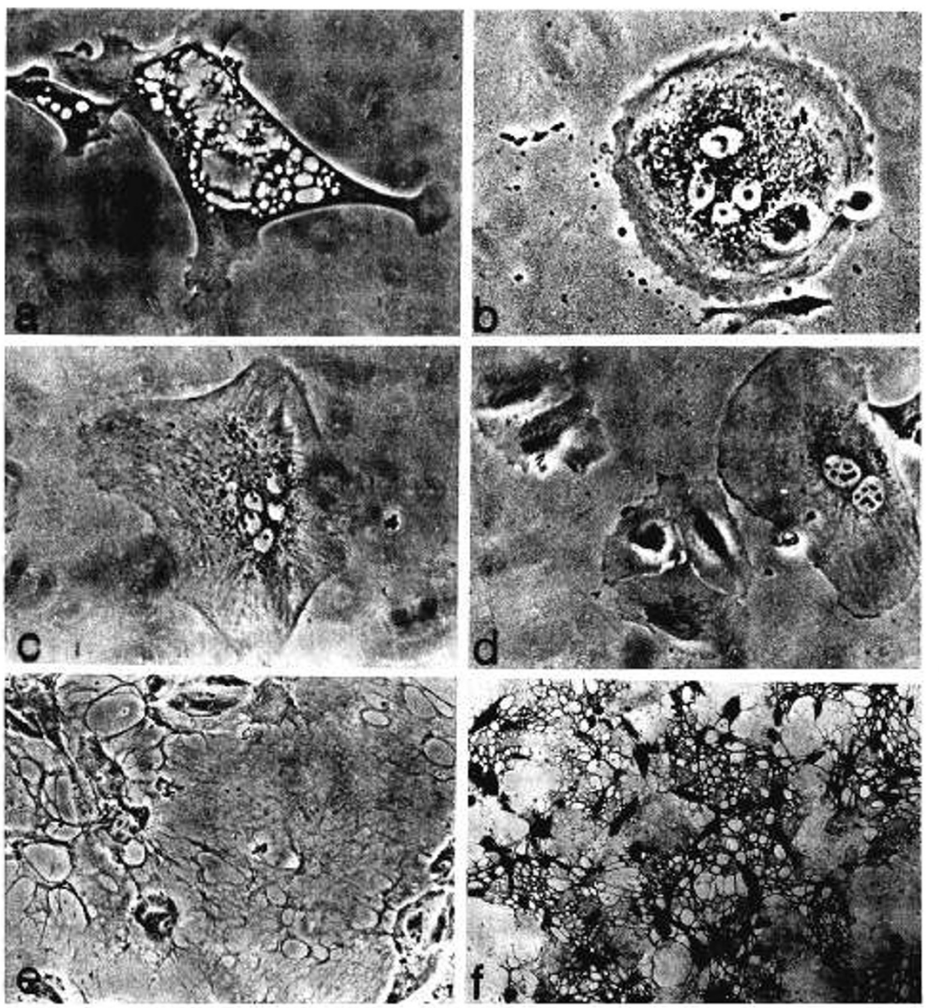

Fig. 2. Examples of nonviable cells (abortive colony formation), and expression of a differentiated state. *a:* vacuolization; *b, c:* micronucleation and accumulation of perinuclear granules; *d:* binucleation; *e:* extracellular substance formation; *f:* periodic acid-schiff-alcian blue stain of *e*. *a–d:* phase contrast, × 123; *e:* × 63; *f:* × 25.

Table 1. *Summary of clonal growth (dilute plating assay)*[1]

Case	Clones/ml total[2]	AF[3] > 20d	Clones case	F	Other AF	Other E	Other X[4]
14	2.4	0.6	13	2	2	7	2
16	5.3	0.75	20	1	9	7	3
17	6.8	1.75	27		23	1	3
18	4.4	2.22	20		17		3
20	2.3	0.25	10		4	2	4
21	2.0	0.0	6		1	5	
24	4.3	1.43	15	2	4	9	
25	7.3	6.0	33	13	14	4	2
26	5.0	3.0	21		15	6	
27	3.5	2.0	15	3	9	3	
28	6.0	4.5	24		22	2	
29	2.5	1.0	10		8	2	
30	0.75	0.25	3		2	1	
31	3.0	1.75	12		11	1	
33	1.5	0.5	6		4	2	
34	2.25	0.75	9	3	2	4	
35	1.75	0.75	7		6	1	
36	2.75	1.5	11		10	1	
37	4.25	0.75	21		15	6	
38	1.0	0.5	5		2	3	
Σ20	3.45$\bar{x}$	1.51$\bar{x}$	Σ288	Σ24	Σ180	Σ67	Σ17

[1] Σ: total; $\bar{x}$: mean; AF: amniotic fluid cells; E: epithelioid cells; CPD: replicative growth potential (cumulative population doublings).

[2] Initial colony counts.

[3] Numbers of clones achieving more than 20 CPD (> 20d).

[4] Colonies which could not be classified at the initial examination.

became clear that the great majority of these primary clones were sufficiently different to warrant a separate classification; we chose the noncommital designation of "AF cells" (amniotic fluid cells). With the untrained eye, these differences were comparatively subtle; in fact, in the first several cases, we were unable to classify a small proportion of clones. With experience however, it became increasingly clear that AF cultures (*1*) were more pleomorphic, (*2*) had a higher proportion of cells with irregular cytoplasmic outlines and extensive "ruffling" of membranes, (*3*) had a smaller proportion of bipolar, spindle-shaped cells, (*4*) had a higher proportion of cells with large and irregular nuclei or with multinucleation, and (*5*) tended to form less clear-cut parallel arrays at confluency. None of these properties, taken individually, would permit a definite differentiation from F type cells, especially since the morphology varied as a function of clonal age (Fig. 3). However, the overall "gestalt," together with a characteristic megaclone pattern (see below), were diagnostic. A third principal category, the E type cell (Fig. 4), is easily distinguished from F cells (4, 8; 25), but could be confused with AF cultures especially when the latter approach senescence. In quantitative terms, AF clones were the most frequent in our assay (66.5%), followed by E (24.7%) and F (8.8%) clones. The latter occurred in only 6 out of 20 samples (Table 1).

SUBCLONAL GROWTH

The cellular morphologies described for primary clones of amniotic fluid cells were reproduced upon subclonal analysis. F subclones tended to exhibit a fairly monotonous pattern of parallel arrays and "streaming" at all stages of clonal formation, while the AF clones typically showed a high degree of cellular pleomorphism and less "organized" growth. With experience, this "constitutional" heterogeneity could be differentiated from that attributable to clonal senescence common to all "hyperplastoid" (16) cell types.

The subcloning efficiencies of F and AF primary clones were extremely variable (range: 0–14%). Although there seemed to be a positive correlation between the subcloning efficiency of an AF primary clone and its CPD, no such correlation was apparent for the F type cultures (Fig. 5).

REPLICATIVE LIFE-SPANS

The statistical distributions of replicative life-spans for all three classes of cultures is shown in Figure 6. Although the sample of F clones is small, it is clear from Figure 6 that they were long-lived (mean of 52 CPD) in comparison with the E types and the majority of AF types, with which there is some overlap. The comparatively great growth potential of F clones was corroborated by the fact that they yielded the largest subclones and megaclones. E type primary clones had the lowest longevity, with a mean of only 14.5 CPD; these cells were also more resistant to trypsinization. It is our impression that many good growing E type colonies, the replicative life-spans of which we here report to be sharply limited, did not recover fully from the subculturing procedure and failed to resume vigorous growth after dispersal.

MEGACLONES

After 20 days of growth, a very striking and characteristic macroscopic and microscopic architecture of "tissue structure" is observed in fibroblast megaclones (Fig. 7, *a–c*) (18). This morphology is in striking contrast to what was observed with megaclones of AF cells (Fig. 7, *d–f*). The latter form colonies with large, densely stained cores of multilayered cells and sharply defined, much narrower peripheral "growth zones" consisting of pleomorphic cell monolayers. The resulting pattern is more of a bulls-eye type than of the "radial rib" type observed with F clones. The bulls-eye pattern is also found in E colonies which achieve the size of megaclones (Fig. 7, *g–i*); the core bulls-eye of an E type tends to be smaller in megaclones, the marginal growth zone being much more extended. Figure 8 illustrates the distinctive character of such megaclone margins.

Quantitative data relating megaclone dimensions to the number of cells per clone confirmed that E cells tended to form larger colonies with fewer cells compared to the other types (Fig. 9). However, in one of the nine samples used for these assays, we observed what we interpreted to be E type colonies with unusually high cell densities and unusually vigorous growth. In contrast to the other samples which gave rise to an average of 13.8 colonies ($>10^3$ cells)/assay, a total of 52 such clones developed in this exceptional sample; while 40 of these appeared to be E-like at the single cell level, their overall megaclone morphologies were intermediate between E and AF (Fig. 7, *j–l*). Regression analysis of clonal cell densities for these atypical (AFE) cells (Fig. 10) revealed a least squares line of best fit significantly different from that of E type cells (Fig. 9) ($p < 0.001$). Six of the megaclones from this sample achieved at least 15 CPD within the 20-day period, which indicated a high growth potential.

CYTOGENETICS

The karyotype was normal diploid in all cases. The frequencies of cytogenetic aberrations were functions of clonal type and stage of culture; details will be reported separately. In three different cases F clones had male karyotypes, thus ruling out maternal origin.

FLOW-CYTOPHOTOMETRIC ANALYSIS

The most convenient and the least subjective way of differentiation between the clonal types was by flow-cyto-

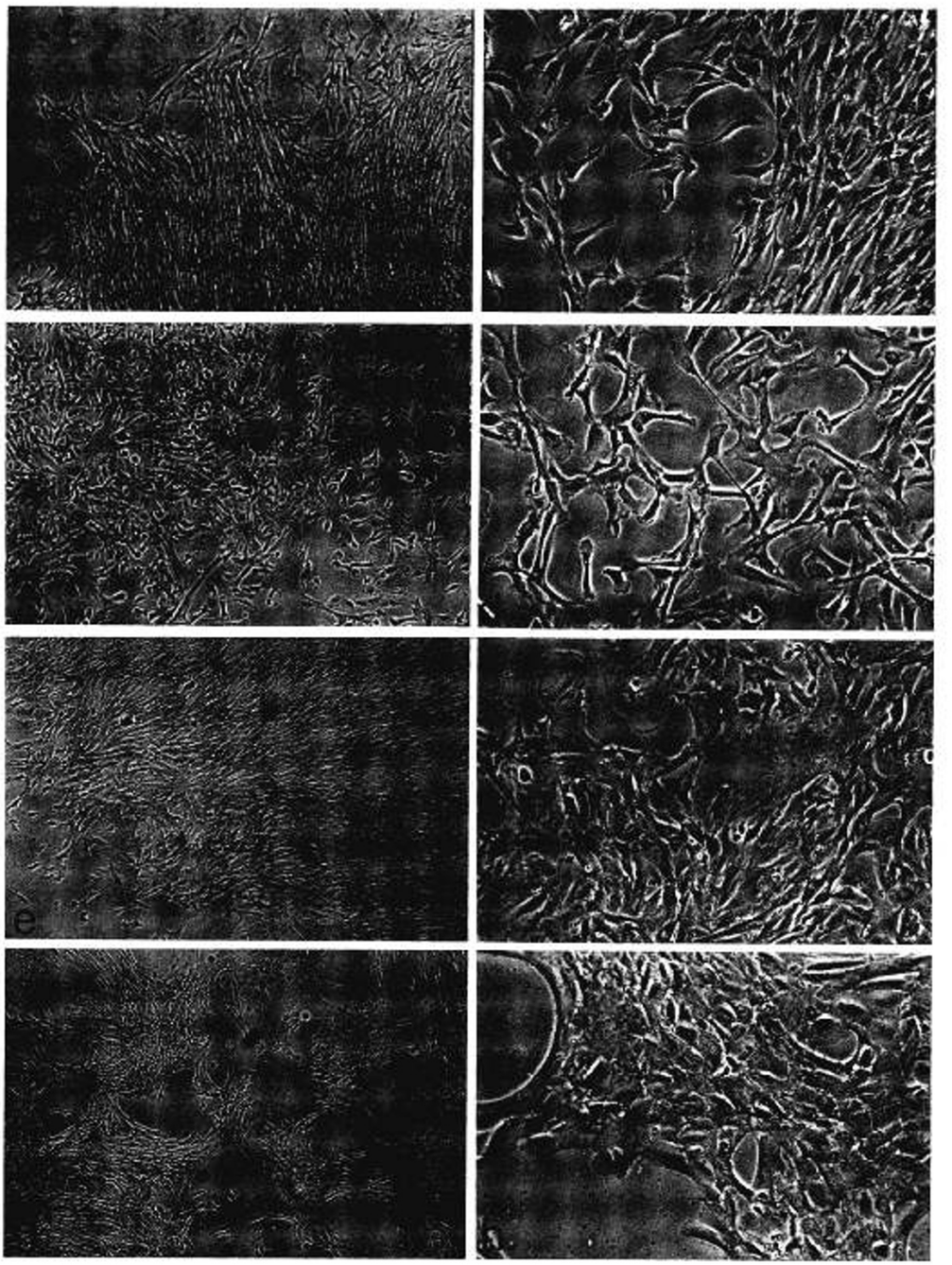

Fig. 3. Living primary clones of amniotic fluid cells: superficial similarity between amniotic fluid (AF) and fibroblast-like (F) types, and morphologic changes during *in vitro* senescence of AF cells. *a,b:* F cells, *c,d:* AF, primary clone, *day 8; e,f: day 16; g,h:* >34 CPD (replicative growth potential). *Left column:* × 25, *right column:* phase contrast, × 63.

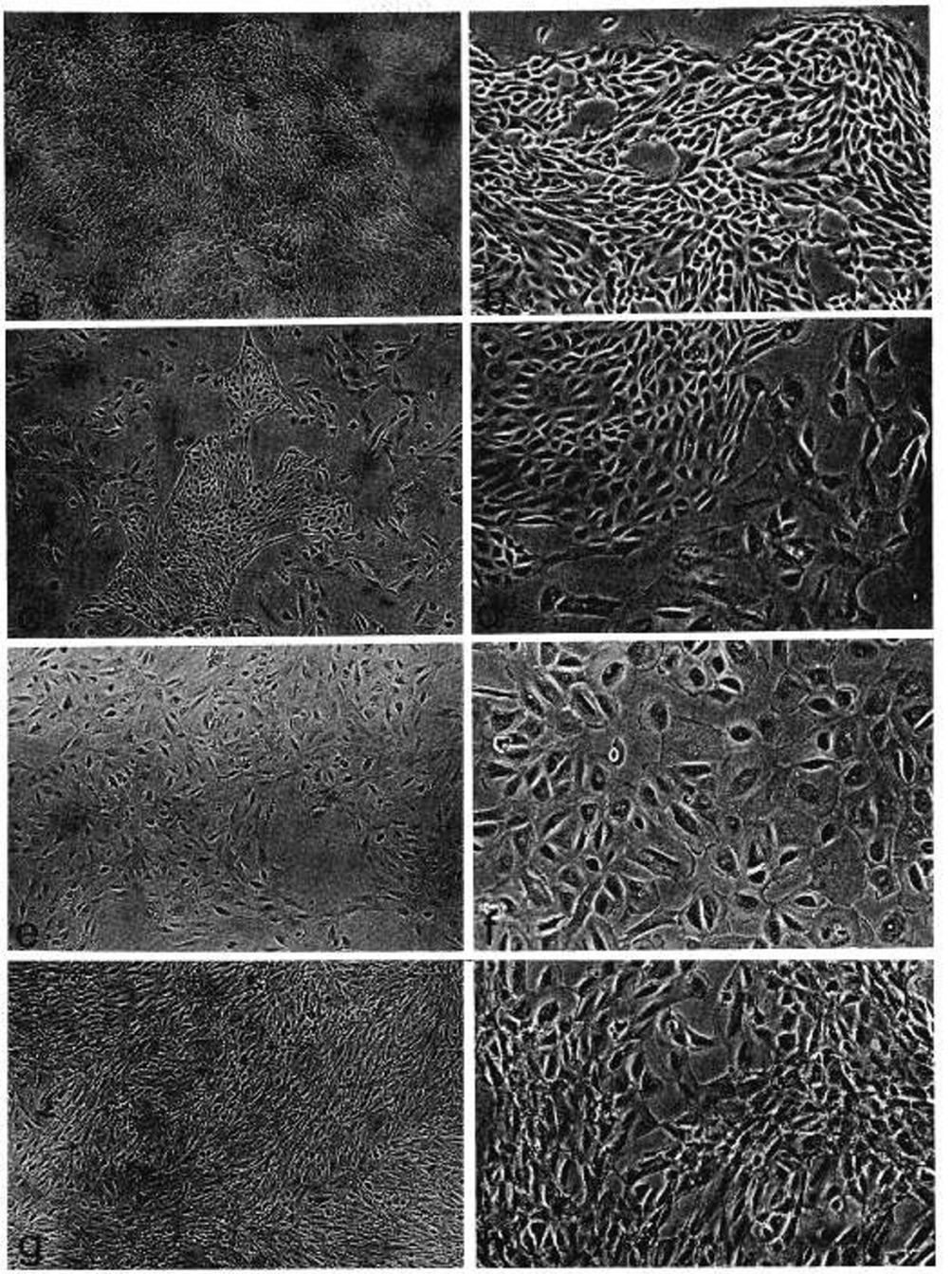

Fig. 4. Living primary clones of epithelioid (E) type cells. *a,b:* clonal organization arising from an intact sheet of approximately 250 cells (3 days after incubation); *c,d:* remnants of the sheet and growth of larger cells in its neighborhood (*day 14*); *e,f:* primary (senescing?) E type colong, *day 26*, attempt to passage failed; *g,h:* confluent areas of vigorous E type colonies. *Left column:* × 25, *right column:* phase contrast, × 63.

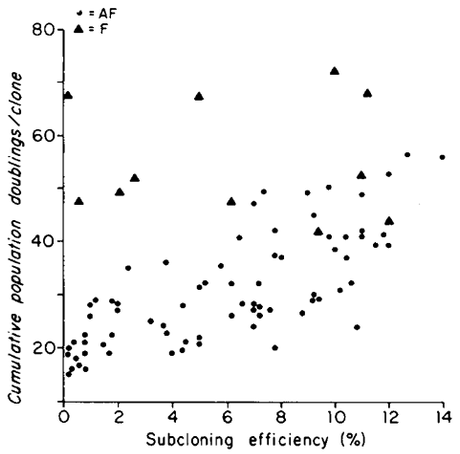

Fig. 5. Prediction of replicative life-spans from subcloning efficiencies: lack of correlation in F type cultures. For definitions of symbols, see legends to Figures 3 and 4.

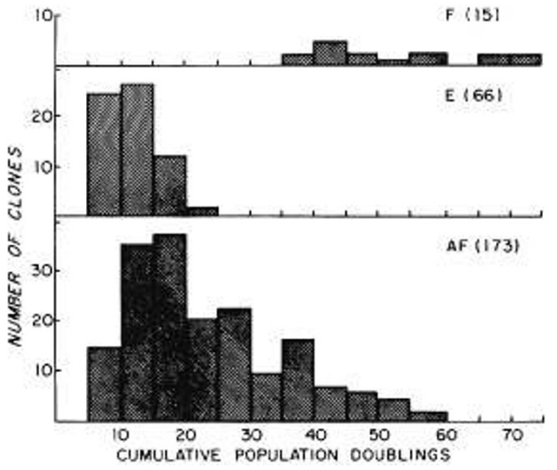

Fig. 6. Replicative life-spans of the three classes of amniotic fluid clones. Number of total clones senesced is given in parentheses. For definitions of symbols, see legends to Figures 3 and 4.

photometric analysis (28) (Fig. 11). Reproducible curves were obtained from actively replicating cultures using standardized harvesting procedures. Surprisingly, E-type curves showed the highest coefficients of variation, in contrast to their comparatively uniform *in situ* appearance.

SUMMARY OF CONTRASTING PROPERTIES OF F, AF, AND E CELLS

Table 2 emphasizes the fact that most of the diagnostic criteria which differentiated our classes of amniotic fluid cells were individually nondefinitive. Exceptions were (*1*) the gross morphology of mature F clones, (*2*) the difference in replicative life-spans between E and F cultures, (*3*) the correlation between subcloning efficiencies and replicative life-spans, and (*4*) the differences in light-scattering properties between F, AF, and E cultures.

DISCUSSION

Most workers in prenatal diagnosis have experienced the morphologic and kinetic heterogeneity of cultivated amniotic fluid cells to the degree of, for example, Uhlendorf's documentation (25). Melancon *et al.* (19) and Gerbie *et al.* (4) have combined morphologic and biochemical variables to establish evidence of two principal cell types which occur in amniotic fluid cell cultures: fibroblasts and epithelial-like cells. The implication has been that the fibroblasts are comparable with skin fibroblasts—prototype cultures studied by biochemical geneticists for many years. In view of our results, it is possible that the "fibroblasts" of these and other workers were what we call AF cells. We do not dismiss the possibility, however, that even subtle differences in media composition could influence the outcome of the competitive replication of the various cell types, so that some laboratories may in fact be dealing with what we have referred to as F type cells. In our hands, these are clearly in the minority and are typically absent altogether in small (3–5 ml) samples of amniotic fluid. If it is assumed that this is the usual situation, what are the implications for prenatal diagnosis?

With respect to the diagnosis of inborn errors involving "household" proteins (3), the diagnosis of severe homozygous deficiency is likely to be comparatively straightforward, regardless of the cell type. This would be the case, for example, in the classic Lesch-Nyhan syndrome (2). For each such enzyme, however, it would have to be established to what extent the specific activity varied as a function of cell type, since even modest differences could confuse the diagnosis of less extreme homozygous deficiencies and of heterozygosity (8). Although the latter would certainly not constitute an indication for selective abortion in typical autosomal recessive disorders, it would be important in subsequent medical counseling.

In the case of the "luxury" proteins (3), knowledge of the cell type could be crucial. This would be the case for both the constitutive levels of certain enzymes and the extent to which enzymes might be inducible (24). Knowledge of the *in vivo* cell of origin of the respective cultures might help provide clues as to which luxury proteins are likely to be expressed or inducible. As an extension of the cytologic work of Casadei *et al.* (1), we are therefore investigating the characteristics of

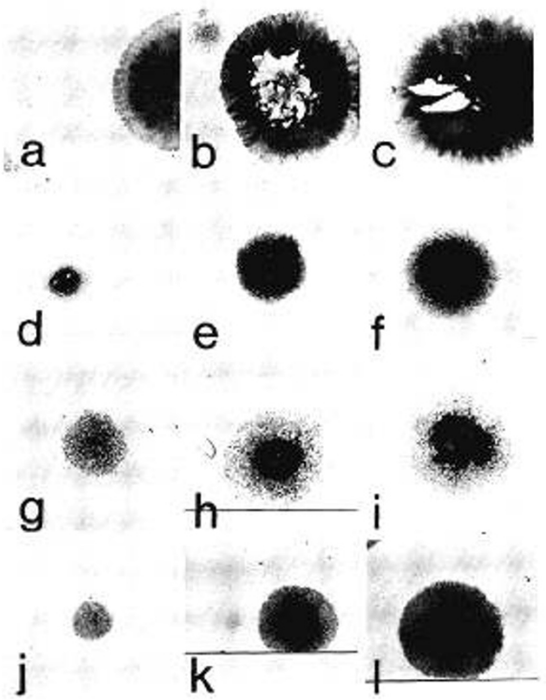

Fig. 7. (*top*). Examples of "megaclones" after a standard 20-day incubation period. *a–c:* F type; the light center areas represent damage caused by prolonged confluence. *d–f:* AF types, bulls-eye pattern; *g–i:* E types (note wider marginal zones as compared with AF); *j–l:* exceptional strain (AC 44) with predominantly E type clones of less distinct megaclone characteristics. Crystal violet, actual size. For definitions of symbols, see legends to Figures 3 and 4.

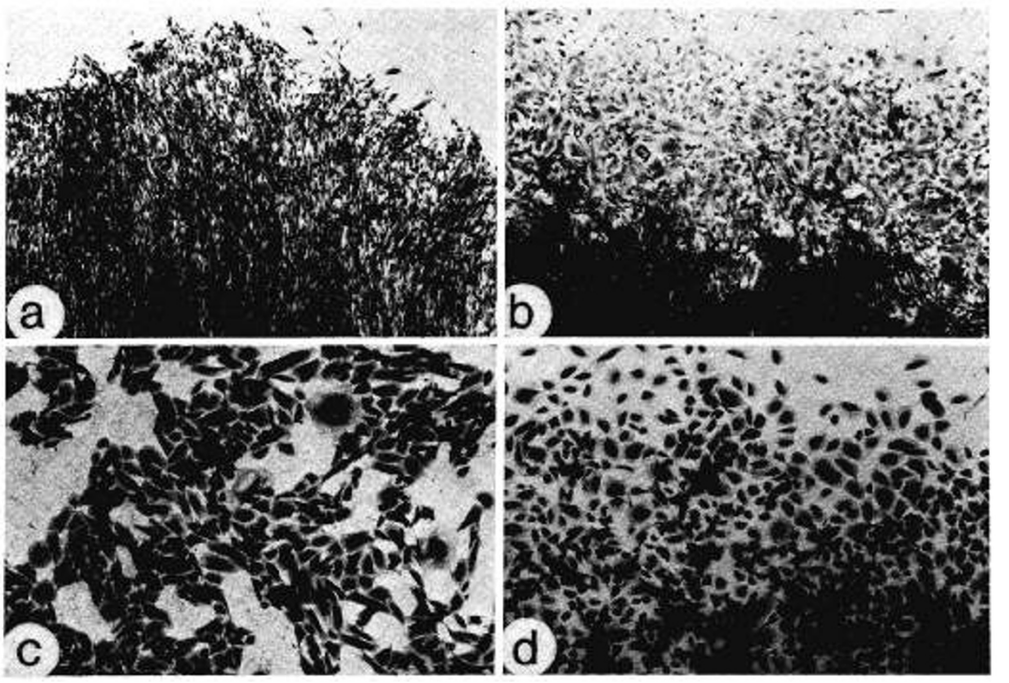

Fig. 8 (*bottom*). Microscopic appearance of megaclone margins. *a:* F type; *b:* AF type; *c, d:* E types. All × 40. For definitions of symbols, see legends to Figures 3 and 4.

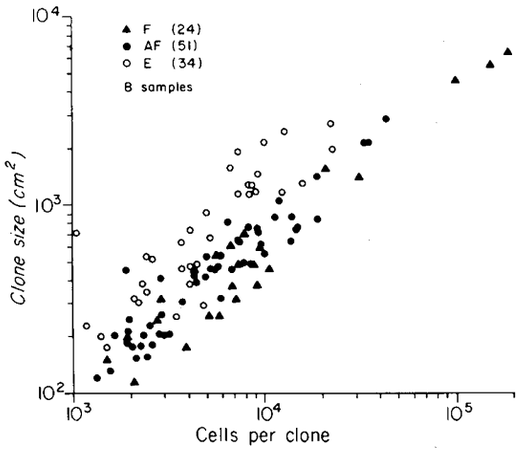

Fig. 9. Relation between megaclone size (total surface area in cm^2 at 55-fold) and number of cells perclone. Data are pooled from 8 different samples. (*F*): $r^2 = 0.93$; $y = -0.47 + 0.81x$; (*AF*): $r^2 = 0.88$; $y = -0.37 + 0.80x$; (*E*): $r^2 = 0.72$; $y = -0.08 + 0.79x$. r^2 = Pearson product-moment correlation coefficient, squared; y = least squares line of best fit. For definitions of symbols, see legends to Figures 3 and 4.

clonable cells derived from specific amniotic and fetal tissues and fluids.

We could not detect any correlation between the initial cell counts and primary cloning efficiencies in our amniotic fluid samples. "Viability" counts as determined by dye exclusion (6, 21) are therefore probably not useful in the prediction of successful clonal growth. The mass cultures which eventually emerge from diagnostic amniocentesis are typically derived from only a few clones and, in some cases, may be derived from a single clone (25). The clone which evolves as the predominant contributor to the mass culture may not be representative of the majority of cells present during early stages of culture. For example, a rare F type cell might have selective growth advantage over numerous E type cells. We should therefore be cautious in assuming that the mass cultures are always representative of the fetus. Even clonal analysis (22) might not be fully reliable, since, as a result of some mutational or recombinational event *in vitro* or *in vivo* (15), a minor mosaic genotype could develop and be greatly amplified by clonal selection. In the clonal proliferation of skin fibroblast-like cells, there is continual selection for progency with the greatest growth potential (18). We therefore believe that attempts to carry out prenatal diagnosis via direct biopsy of blood or tissue should be encouraged (10, 13, 26).

SUMMARY

Clonable amniotic fluid cells were classified into three principal categories, F, AF, and E, on the basis of morphologic and growth kinetic criteria. To our knowledge, the most frequent category (AF) has not been characterized previously and separated from "fibroblasts," which are comparatively rare in such cultures, but have the greatest growth potential.

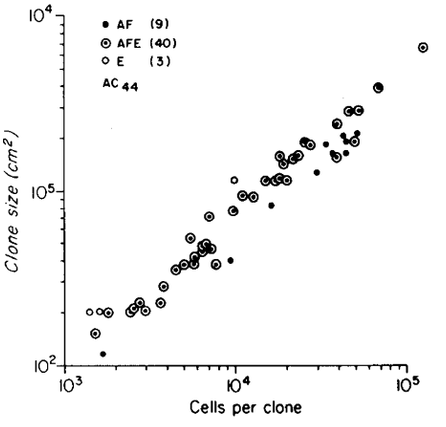

Fig. 10. Relation between clone dimensions and number of cells per clone; data from the exceptional sample (AC_{44}); *AFE:* exceptional colonies. (*AF*): $r^2 = 0.98$; $y = -0.86 + 0.89x$; (*AFE*): $r^2 = 0.97$; $y = -0.56 + 0.85x$. r^2 and y as in Figure 9. For definitions of symbols, see legends to Figures 3 and 4.

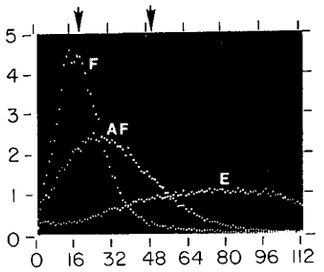

Fig. 11. Optical properties of F, AF, and E suspension (6×10^4 particles each). Reproduction of the original oscilloscope display; *ordinate:* counts $\times 10^3$; *abscissa:* channel number, proportional to light scatter and/or absorbance. The arrows indicate the positions of peaks of 9.7-μm (*left*) and 15-μm (*right*) diameter polystyrene particles (32) under settings identical with those used for the cells. Confluent monolayers were dissociated with trypsin, after two washes with phosphate-buffered saline. Equal amounts of fixative (20% formaldehyde in phosphate-buttered saline: methanol = 1/1) were added to the trypsin-cell suspension in 12 ml conical centrifuge tubes. After centrifugation, cells were resuspended in total fixative and stored overnight at 4°C. They were washed twice with glass-distilled H_2O before counting. F and AF displays are from sister cultures of one sample (26 CPD); E is from another sample (22 CPD). For definitions of symbols, see legends to Figures 3 and 4.

Table 2. *Summary of contrasting properties of fibroblast-like (F), amniotic fluid (AF), and epithelioid (E) cells.*

Clonal "ribbing" pattern	F
Subcloning efficiency ≠ FCPD[1]	F
Parallel arrays at confluency	F > AF
Spindle configuration	F > AF > E
Mean CPD	F > AF > E
Uniform light scattering[2]	F > AF > E
Clonal bulleye pattern	AF & E
Indistinct cytoplasmic outlines	AF > F
Loose net-like growth[3]	AF > F
Cellular pheomorphism	AF > E > F
Trypsin resistance	E
Sheet-like coherence	E
Epithelioid configuration	E > AF > F
Large cells or nuclei	E > AF > F
Multinucleation	E > AF > F

[1] ≠ FCPD symbolizes lack of proportionality to cumulative population doublings.

[2] Cytograf (28).

[3] Characteristic of early and late phases of growth.

NOTE ADDED IN PROOF

Aspects of clonal heterogeneity of amniotic fluid cell cultures have been similarly emphasized by Werner Schmid (Triangle *11*, 91 (1972)).

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