Growth of HeLa Cells in Human Adult and Bovine Fetal Serum Medium.* (27743)

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A requirement for both human adult and bovine fetal serums as medium supplement for colonial growth of a line of continuously propagable cells has been reported(1), and effects of these serums on morphological and other properties of newly established human cell strains have been described(2). Since differing effects of the serums were observed with these cells, it was of interest to determine effects on growth of a cell strain known to many investigators by its substrains. This report describes growth of HeLa cells in medium supplemented with human adult and bovine fetal serums singly and in combination. Differing effects of the serums on cell size, colony morphology and cell viability may ultimately be significant in approach to elucidation of factors controlling form and multiplication of animal cells.

Materials and methods. HeLa cells, line 229, from the strain of human cervical carcinoma propagated by tryptic dispersion by Scherer, Syverton and Gey(3), were received from frozen storage in glycerol medium as passage 8. This line had been shown free of contamination by pleuropneumonia-like organisms by bacteriologic test(4) and it was fully poliovirus-susceptible. Squash preparations of propagated thawed cells were made after hypotonic saline treatment, acetic-acid-alcohol fixation and aceto-orcein staining. Chromosome counts ranged from 78 to 124 per cell.

Salt solutions used for rinsing cultures for dispersal and for diluting cell suspensions respectively were buffered with 0.6 mM (millimolar) phosphate and 6 mM phosphite at pH 7.3 and 7.8; these solutions contained 120 mM sodium chloride, 6 mM potassium chloride, and low concentrations (0.1 mM) of calcium and magnesium chlorides. Diluting medium was supplemented with 2% (v/v) bovine fetal serum. Basal medium contained lower concentrations of sodium and potassium chlorides (100 and 5 mM), 1.0 mM calcium and magnesium chlorides, 2 mM phosphate and 20 mM phosphite adjusted to pH 7.3, fructose (10 mM), glucose (0.1 mM), neomycin sulfate (50 μg/ml), and other materials as previously described(1). Vitamins and amino acids of Eagle minimal essential medium also were added (0.2 mM cysteine was substituted for 0.1 mM cystine of the original formula(5)). Bovine fetal serum (Colorado Serum Co., Denver) and pooled human adult serum (blood group A positive) were filtered through ultrafine sintered glass and stored frozen in small lots. Capacity of these serums to support colonial growth of human cells had been verified.

For stock, frozen HeLa cells were thawed rapidly at 37°C and grown in 60 mm Pyrex glass Petri dishes in basal medium supplemented with 10% each of human adult and bovine fetal serums (passage 9). Dishes for cultures were prepared after washing and before rinsing by immersion in 10 mM tetrasodium ethylenediamine tetraacetate (EDTA). Stock and experimental cultures were dispersed by rinsing and treatment for 5 to 10 minutes with 0.05% (w/v) trypsin (Nutritional Biochemicals Corp. 1-300) in 1.25 mM EDTA solution at pH 7.3. Dispersed cells in diluting medium were diluted further in counting fluid(2) for counting in a Coulter Model B electronic cell counter. Appropriately inoculated cultures were incubated at 36.5°C in a humidified incubator gassed continuously with 2.5% carbon dioxide in air. Mediums were supplemented with sodium bicarbonate solution to yield pH 7.3 in this atmosphere.

Size distributions of dispersed cells in counting fluid were determined by use of the Coulter automatic size-distribution plotter, calibrated with ragweed pollen of 20μ nominal mean diameter. For colony counting and morphol-

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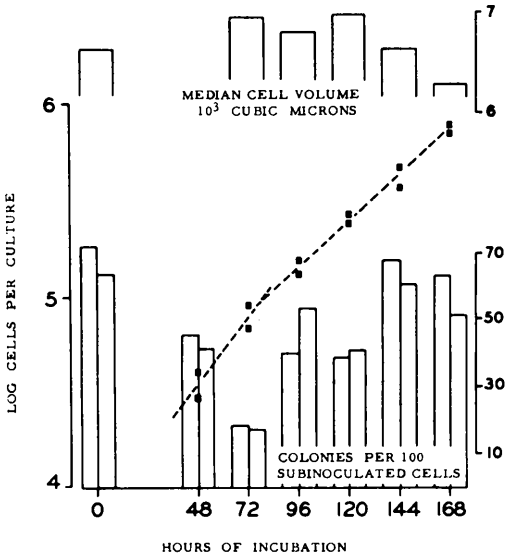

FIG. 1. Growth of HeLa cells, line 229, in stationary plane culture in vitamin-amino-acid medium supplemented with 10% each of human adult and bovine fetal serum. Duplicate Petri dishes with 5 ml of medium were inoculated with 10^4 trypsin-EDTA-dispersed cells, and incubated at 36.5°C; the medium was not changed during growth. Harvested cells were sized electronically, and viability was determined by formation of colonies from 100 cells subinoculated into the same medium. Dashed line shows total number of cells accumulated in culture (left scale); top bars show median cell volume (right top scale); and bottom bars show viability of harvested cells by plating efficiencies for duplicate subcultures (right bottom scale).

ogical record, experimental cultures after incubation were stained with Wright's stain.

Experimental cultures were prepared as passage 10 of the stock HeLa, the second passage of the thawed cells. First, 10^4 trypsin-EDTA-dispersed cells were inoculated into replicate dishes containing 5 ml each of basal medium supplemented with 10% human adult and 10% bovine fetal serum. After 2 days of incubation and daily thereafter, duplicate cultures were rinsed, dispersed, and sampled. One sample was used for counting and sizing of cells; from the other sample, 100 cells were inoculated per dish into mixed serum medium in duplicate. The original medium on the test cultures was not changed during the period of observation.

Second, 10^2 dispersed cells of passage 10 were inoculated into sets of 4 dishes containing 5 ml of basal medium supplemented respectively with 10% each of human adult and bovine fetal serums, 20% human adult serum, or 20% bovine fetal serum. After 7 days of incubation without medium change, 2 cultures of each set were dispersed for cell counting and sizing, and 2 cultures were stained for colony counts.

From the size distribution recorded by the Coulter plotter, median cell volumes were determined by plotting cumulative per cent of sampled cells per volume range on arithmetic-probability grid.

Results. Growth of HeLa cells in mixed serum medium, as revealed by change with time in cell number, median cell volume, and viability (colonies generated per 100 subcultured cells), is shown in Fig. 1. Although the increase in cell number did not suggest departure from an exponential course of accumulation, the regression appeared biphasic. About midway in the course of growth, the recorded median cell volume was greater than at beginning or end of the period of observation, and similarly cell viability was less. Effects on culture parameters of the serum components of the medium are shown in Table I, where efficiency of colony formation is compared with median cell volume after incubation, and with growth efficiency calculated by dividing the

TABLE I. Effect of Medium Serum on Colonial Growth of HeLa Cells.

Serum supplement, %	Efficiency of plating*	Growth efficiency†	Corrected growth efficiency‡	Median cell volume, μ^3
Human adult, 10 with bovine fetal, 10	71, 63	74.5	111	6000
Human adult, 20	51, 20	39.0	106	7425
Bovine fetal, 20	81, 84	60.8	74	3670

* Cultures were incubated for 7 days without medium change. Efficiency of plating: No. of colonies per dish / No. of inoculated cells, × 100.

† Growth efficiency: No. of cells per dish after incubation / No. of cells inoculated.

‡ Corrected growth efficiency: growth efficiency / plating efficiency as fraction (*i.e.*, No. of cells produced per viable cell inoculated).

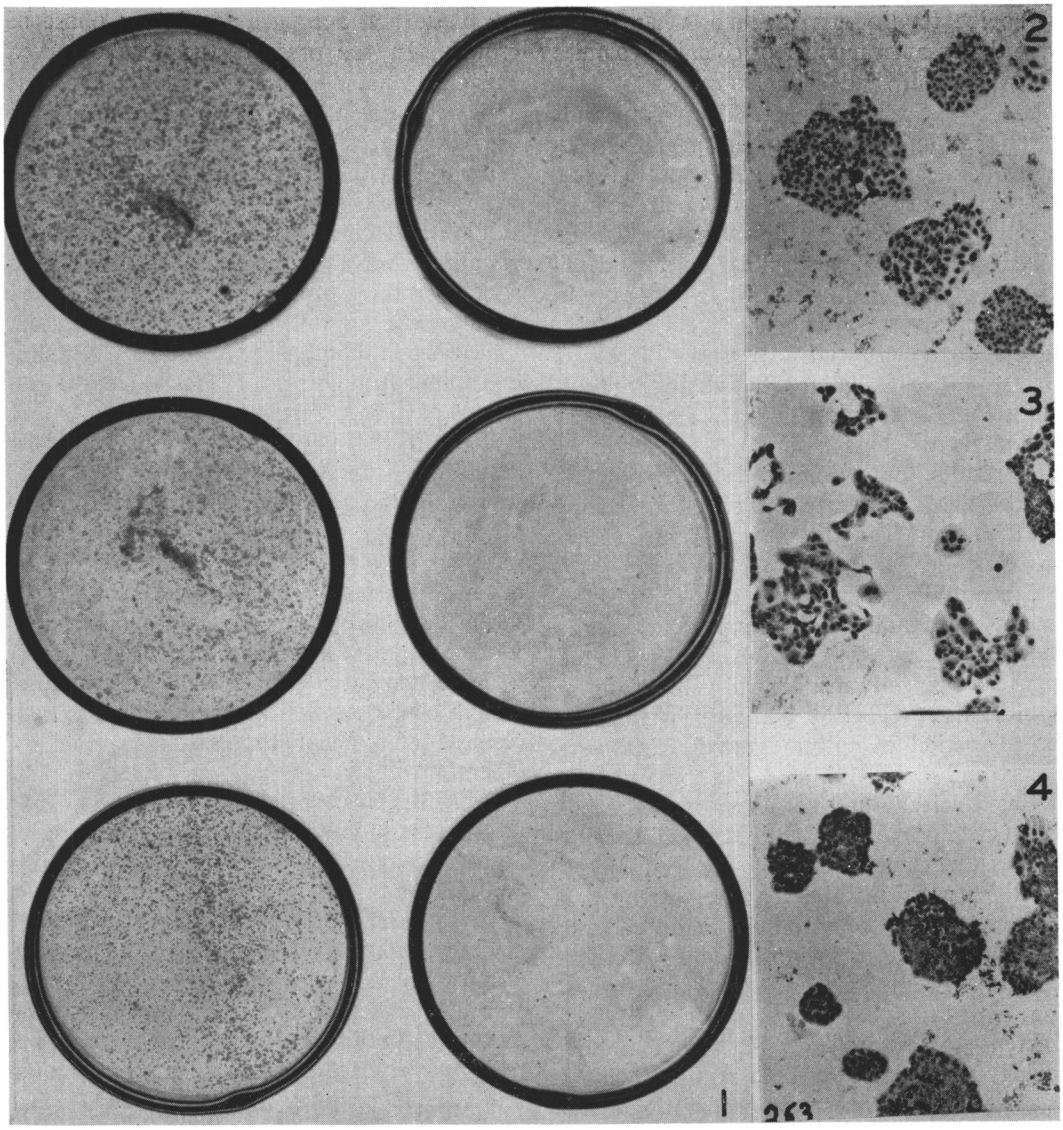

PLATE 1. Colonial growth of line 229 HeLa cells in vitamin-amino-acid medium supplemented with 10% each of human adult and bovine fetal serums (top horizontal row), 20% human adult serum only (middle row), and 20% bovine fetal serum only (bottom row), 1×. Cultures in left column were inoculated each with 10^4 cells, dishes in right column with 10^2 cells. Colonies stained with Wright's stain.

PLATE 2. Individual colonies from medium with mixed serums, 42×.

PLATE 3. Individual colonies from medium with human adult serum only, 42×.

PLATE 4. Individual colonies from medium with bovine fetal serum only, 42×.

average number of cells yielded by 2 cultures by the number of cells inoculated. The lowest yield and plating efficiency were associated with yield of largest cells, and essentially the reverse. The largest and least viable cells were yielded by the human adult serum medium, while the smallest and most viable cells were yielded by the bovine fetal serum medium. It also is apparent from the table that correction of the growth efficiency for plating efficiency would suggest that the least population increase per viable cell occurred in the bovine fetal serum medium, while the increase per viable cell was about the same in human adult or mixed serum medium. Morphologic aspects of these results are shown in Plate 1,

In bovine fetal serum medium, where there were the greatest number of colonies, colonies all were small, and cell arrangement within colonies was very compact. In human adult serum medium some of the colonies were larger, and cell arrangement appeared less compact by reason of greater cytoplasmic area of cells. The largest colonies were seen in the mixed serum medium; cellular form and arrangement appeared intermediate to that seen in the single serum mediums. As evident from the photographs, in all 3 mediums there was some variation in cellular morphology in colonies; this variation within a culture appeared greatest in the human serum medium. From these results it was concluded that the apparent cell-size variation observed in the course of HeLa growth in the mixed-serum medium was real.

Discussion. In medium containing both human adult and bovine fetal serum, while multiplying exponentially HeLa cells exhibited apparent change in median cell volume and in viability as revealed by plating efficiency. In the midperiod of culture growth, cells were largest and viability lowest. As measured by plating efficiency and corrected growth efficiency (yield of cells per inoculated viable cell), the 2 component serums of the medium affected growth differently, as well as median cell size. Contrasted with human adult serum, the bovine fetal serum appeared to decrease cell size, increase frequency of colony formation, and decrease yield per viable cell. The differences were reflected in cellular morphology and colony form. It was not evident whether the effects represented differing cellular responses, and/or responses of different cells. Mixed serum medium combined the advantages of single serums for growth of the HeLa cells. In these studies it was observed also that cells grown in bovine fetal serum medium were dispersed poorly by 0.05% trypsin unless supplemented with EDTA. Conversely human-serum-grown cells were readily detached from glass with trypsin, and dispersal of cell sheets was not particularly aided by EDTA.

Changes in mean cell volume during growth of L-strain mouse cells in suspension, for example, were observed by Merchant *et al.* (6); these changes were most marked in the lag phase and approach to plateau phase of growth. In the present study we observed changes in population properties during what appeared to be a biphasic log phase of culture growth. Since the HeLa cells were aneuploid and varied with respect to number of chromosomes per cell, the variation seen both in cell volume and colonial morphology as well as viability, and the effect of growth-medium serums on these properties, may have resulted from genetic as well as physiologic variation. Variations in morphology of colonies, cell size, and other properties of HeLa clones and strains have been described by others(7-9). It is doubtful that effects of the serums resulted only from selective growth of stable genetic variants in the donor population, because combined plating efficiencies of cells in single-serum mediums exceeded plating efficiency in mixed-serum medium, and colonies in mixed-serum medium were not simply a mixture of forms seen in the single-serum mediums. These findings emphasize the possible influence of growth course as well as medium serum supplement on cellular properties, as seen by others(9).

While the effects of human adult and bovine fetal serum in culture medium for HeLa cells observed here must be referred to the particular serum lots used, it is shown that at least some samples of the serums can be supplementary, and that the usefulness of such mixed serum supplement need not depend on dilution or neutralization of "toxic" effects of the two serums, as the word usually is understood.

Summary. HeLa cells cultivated in amino acid-vitamin medium supplemented with human adult and bovine fetal serum grew exponentially during 5 days of incubation in unchanged medium from 48 hours after inoculation of cultures. Change in growth rate was associated with change in viability and median cell volume. Colonial cultures grown in mixed and single-serum mediums exhibited differing effects on median cell volume, plating efficiency and cell yield per viable inoculated cell, and on cellular and colonial morphology.

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The Importance of Sample Size in Studies Based upon the Serologic Classification of *Escherichia coli.** (27744)

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The ability of specific antisera to agglutinate coliform bacteria was recognized prior to 1900. The importance of this observation could not be fully realized until a serologic classification based upon the presence of heat stable O and heat labile K somatic antigens was developed by Kauffmann(1). The original scheme has been extended by many other workers until it is now possible to identify some 138 different O groups of *Escherichia coli.* The importance of this technic has been reflected in contributions to our understanding of the association of *E. coli* of specific O groups with intestinal and extraintestinal diseases(1,2,3,4,5,6). Additionally, it has been shown(3,4,5) that the fecal flora may be composed of a number of different serologic groups of *E. coli* while extraintestinal materials tend to contain only a single serologic group. The relation of sampling technics to total number of groups identifiable in the individual specimens has not been explored. Most investigators have studied only 2 to 4 colonies and rarely as many as 10 per specimen.

During studies of the epidemiology and pathogenesis of infections of the urinary tract, precise information as to the relation of the sample size obtained from specimens from various sources to the total number of groups present was needed. In this paper such information is presented, derived from a systematic study of strains of *E. coli* isolated from stool, urine, vaginal, and blood specimens obtained from diseased and normal patients.

Materials and methods. Clinical materials. Sixty-four stool, 100 urine (containing more than 100,000 organisms per ml), and 50 vaginal specimens were collected from patients with symptomatic infections of the urinary tract. Thirty-six stool samples and 100 urines containing less than 100,000, and usually less than 10,000 *E. coli* per ml, were obtained from students, laboratory personnel, and patients without evidence of active disease of urinary or intestinal tracts. The "clean catch-midstream" voiding technic was used to collect all urine specimens. Urinary tract infection was present in 24 of 35 patients with bacteremia due to *E. coli;* other portals of entry were apparent or could not be determined in the remainder.

Bacteriology. The clinical materials were inoculated on blood, eosin-methylene blue, and MacConkey agar plates. Fifteen to 25 colonies typical of *E. coli* were selected from each stool, 5 from each urine and vaginal, and 2 to 5 from each blood specimen. All strains of *E. coli* studied fermented lactose rapidly, and failed to utilize citrate; most were methyl red and indol positive.

Serologic classification. Unabsorbed sera prepared by immunization of rabbits with standard O groups of *E. coli* were obtained from the Communicable Disease Center, U. S. P. H. S., Atlanta, Ga. Sera for 129 O groups

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