# Cellular Variants for Blood Groups Within an Established Cell Line[1] (34526)

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In earlier studies blood group activity was detected by mixed agglutination on HeLa cells (1, 2). The expression of surface group H on these cells was found to be variable when strains from five different laboratories were tested (2). Thus, the number of positive cells ranged from 30 to 57% among HeLa cell strains derived from five different sources. The above results were obtained in the presence of the same high-titered anti-H reagents, and the cells under study did not appear to be contaminated either with PPLO, bacteria, or other cell lines. Synchrony experiments carried out on cells from one HeLa strain (S-3) indicated that expression of surface group H activity was variable and associated with metabolic phase of the cells. In such experiments populations containing high percentages of mitotic cells were seen to be more reactive than interphase cells (4). The findings, if confirmed, would be consistent with those of others who have observed increases in the content of cell markers in replicating cell systems (5).

The interpretation of these experiments could be complicated by the existence on HeLa cells of more than one group H immunodeterminant corresponding to the activity of ulex anti-H reagent. Thus, Florey found that the mixed agglutination of group H buccal epithelial cells was variable from one individual to another, and that ethanol fractions derived from ulex extract permitted a distinction into several H-reactive antigens (6). If multiple inherited forms of group H reactivity exist on HeLa cells, this fact would need to be recognized, and the antisera used in testing processed accordingly.

An additional possibility to account for HeLa cells apparently unreactive for group H is the occurrence of blood groupless variants, possibly related to the selective loss of cellular enzymes involved in blood-group synthesis. Loss of alkaline phosphatase enzyme in a strain of HeLa cells has been reported (7).

In assessing the alternatives described above, it was felt that studies of HeLa cell group H would be enhanced if the mixed agglutination technique was augmented by the use of fluorescent labeled antibodies. The latter might be expected to provide information about the geographical distribution of antigen in cell clones and on individual cells. Examination of stained clones and their descendants might also provide evidence for the heritable nature of group H variants on cultured cells. The present experiments describe agglutination of HeLa cells by anti-H ulex reagent as a selective method for deriving a population of apparently unreactive HeLa cells which were then cloned and subsequently examined for group H using fluorescent labeled anti-H antibodies.

*Materials and Methods. HeLa cell strain.* The strain HeLa-P was originally obtained from Dr. Theodore Puck, Department of Biophysics, University of Colorado, and was maintained in continuous culture in our laboratories.

*Nutrient media.* Eagle's minimal essential medium (MEM) modified according to Levintow and Darnell (8) was employed for standard maintenance of the HeLa lines. Media contained a final concentration of

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15% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.25 μg/ml fungizone. Inhibition tests, using either whole nutrient medium or fetal calf serum in combination with anti-H extract and group O erythrocytes, failed to reveal any evidence of group H activity in these components.

*Antisera.* High-titered anti-H globulin was prepared from the seeds of the plant *Ulex europeus* using a method described by Boyd (9). Reagents for control experiments were prepared by absorbing 1 vol of antiserum with 2 vol of packed group O erythrocytes.

*Human erythrocytes.* Fresh human group AB and O erythrocytes were prepared as described in a previous publication (3).

*Culture procedures.* HeLa cells grown in suspension cultures were prepared from healthy 4-day-old monolayer cultures which had been prepared as described previously (3). The nutrient medium for this purpose consisted of Eagle's MEM spinner medium with fetal calf serum and antibiotics as indicated above.

*Mixed agglutination.* A modification of the mixed agglutination technique described by Kelus, Gurner, and Coombs (1) was carried out. In controls group AB erythrocytes were utilized in mixture with anti-H-sensitized HeLa cells.

*Fluorescent labeled antibodies.* Ulex anti-H reagent of high titer was prepared using a technique described by Kent (10). The final conjugated globulin possessed titers of 1/256–1/512 when tested against a 2% suspension of group O red cells. Controls consisted of experiments designed to give inhibition of staining by (1) absorption of conjugated anti-H globulin with homologous group erythrocytes; (2) pretreatment of cells with unconjugated anti-H antibodies. (3) neutralization of anti-H with group H-soluble substance. Distinction between tests and controls was found to be improved by absorption of fluorescent labeled antisera with mouse liver powder as described by Coons (11) and Szulman (12). The percentages of stained HeLa cells were compared with percentages of cells uninvolved in mixed agglutination.

*Cloning experiments.* Healthy 4-day-old suspension cultures were trypsinized and counted. The number of cells positive for group H was determined by mixed agglutination. Plastic petri dishes, 60 mm in diameter, were seeded with $10^4$ single cells suspended in Eagle's MEM spinner medium with added fetal calf serum and antibiotics. Petri dish cultures were incubated from 7 to 10 days at 37° in an atmosphere of 5% $CO_2$ and balanced air. In parallel experiments designed to obtain a population of unreactive cells, anti-H ulex extract was added to a suspension culture of HeLa cells, and agglutination was carried out in capped centrifuge tubes under sterile conditions. The supernatant suspension was removed from agglutinates which had sedimented at 30–40 min and followed by centrifugation at 200 rpm. The supernatant cells were counted and cultured in petri dishes in parallel with complete suspensions as described above. At the time that petri dish cultures were prepared, cover slips were also seeded with $10^3$ single cells and permitted to grow in Demuth flasks for 3–4 days at 37° in 5% $CO_2$ and balanced air. Cultures for the presence of bacteria and PPLO were carried out and reported as negative during all phases of this study.

*Fluorescent antibody staining.* Cover slip preparations were washed and stained, along with controls, and viewed under the ultraviolet light microscope. From petri dish cultures, colonies were removed, using sterile precautions, and single-cell suspensions were prepared by trypsinzation. Aliquots were fixed (12), stained, and examined as described above. Subcloning experiments were carried out on cover slips with the remainder of the cell suspensions in the case of positively and negatively reacting clones.

*Results.* Figure 1 depicts a cover slip preparation from the original HeLa cell suspension. A marked variability was seen in the staining properties of these cells. Thus, about four colonies exhibited mild or no evidence of H antigen activity while a group of cells consisting of four or five closely adjacent colonies was H active. Most colonies contained one or two cells which stained much more brightly than the remainder. The

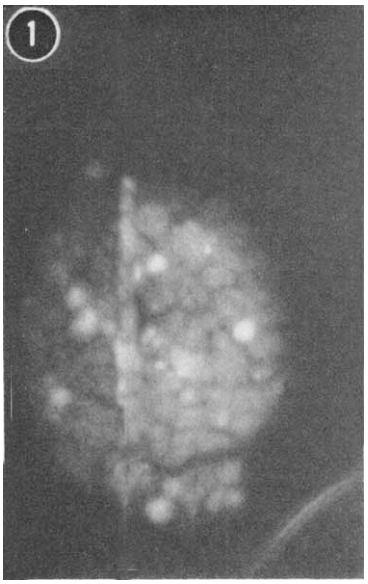


FIG. 1. Cover slip preparation from original HeLa cell suspension. Cells were grown 4 days, then fixed and stained with fluorescent labeled anti-H ulex reagent 250×.

distribution of staining in a few cells was peripheral with a dark interior, but staining within most cells was more or less uniform. The proportion of positively reacting cells was close to the percentage of cells positive by mixed agglutination in the suspension used in the preparation of this cover slip. Thus, approximately two thirds of cells were considered to be positive, and these were descendents from cells 58% positive for group H by the method of mixed agglutination.

Figure 2a indicates the appearance of HeLa cell clones on petri plates 10 days after $10^4$ cells from the original cell suspension had been placed into culture. In Fig. 2b are clones derived from the cellular supernate of the suspension from which group H active cells had first been removed by specific agglutination. Individual colonies were removed from each petri plate using sterile precautions. Individual cell suspensions were prepared from a clone by trypsinization and concentration of the resultant suspension in the centrifuge.

Figure 3 indicates the appearance of an aliquot removed from a treated clone derived from the original cell suspension (Fig. 2a). Practically all of these cells fluoresced brightly when stained with labeled anti-H ulex reagent. A variable distribution within or on cells was observed; that is, some exhibited more or less homogenous fluorescence, whereas others exhibited peripheral but not internal fluorescence. On the other hand, suspensions prepared from 12 clones derived from supernate cells unreactive in mixed agglutination were either entirely negative for group H (Fig. 5), or mixed negative and positive as observed in Fig. 4. A consistent finding was that the intracellular staining pattern differed between individual cells.

The remainder of the cell suspensions not utilized for fluorescent studies was seeded onto cover slips and grown for 4 days as previously described. The resultant cover slip preparations were washed and examined after they had been stained with fluorescent labeled anti-H ulex reagent.

Figure 6 indicates the staining pattern of a fixed (12) cover slip preparation of cells derived from a positively reacting clone. Most of the cells in these colonies stained positively in the presence of fluorescent labeled anti-H ulex reagent. In a similar fashion the cellular progeny of mixed or of negatively reacting populations reflected the composition of cells in the parent clones. Thus, a mixed population reactive for group H was observed on colonies on the cover slip whose

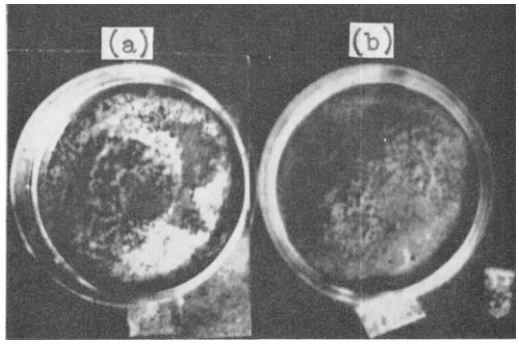


FIG. 2. Appearance on petri plates of 10-day-old HeLa cell clones grown from (a) mixed population with respect to cells containing group H and (b) supernatant cells from the original population whose H-containing cells had been separated by agglutination.

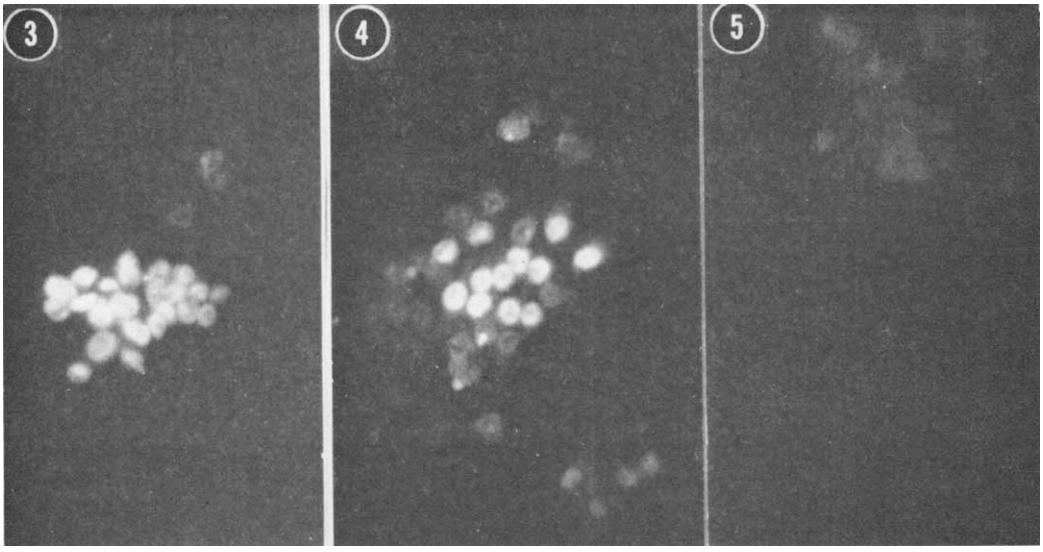


FIG. 3, 4, and 5. Fixed smear preparations of cells from selected clones after removal fom petri plate, disaggregation and concentration, and staining with fluorescent anti-H ulex reagent. 250×. Figure 3 illustrates cells from positively reacting clone derived from original cell suspension; Fig. 4 illustrates mixed positive and negative cell preparation derived from supernatant cells; Fig. 5 illustrates negative cell preparation derived from supernatant cells.

cells were derived from clones of related composition (Fig. 7). In the case of negatively reacting clones, the cellular progeny also did not react or stained only slightly in the presence of labeled ulex extract (Fig. 8).

*Discussion.* Cellular progeny derived by subculture from a strain of HeLa cells gave mixed positive and negative results for blood group H by the methods of mixed agglutination and immunofluorescence. Agglutination of the positively reacting population by anti-H ulex reagent provided a means for selecting from the supernatant fluid cell populations apparently deficient in this blood

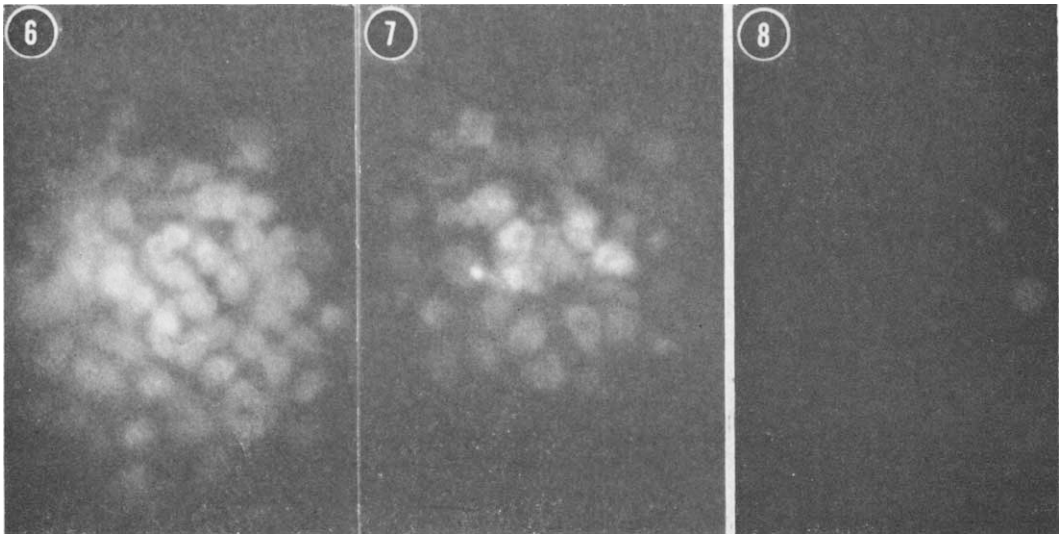


FIG. 6, 7, 8. Cover slip preparations of stained cells derived by subculture from clones on petri cultures. In each case, staining properties resemble those of cells derived from parent clones. 400×.

group. The descendents of these cells, when cloned, yielded serological reactions similar to the parent cells. The results were consistent with the presence in this HeLa strain of at least three cellular populations: (1) cells negative for group H are believed to represent groupless variants whose other characteristics are not incompatible with continued growth; (2) cells positive for group H which apparently retained the original group H immunodeterminant; (3) cells whose progeny consisted of mixed positively and negatively reacting cells, and may reflect partial loss of the H immunodeterminant.

Evidence of the complex nature of the H antigen has been derived from the studies of Florey (6) who utilized ethanol fractions of ulex reagent to define at least two immundeterminants in buccal epithelial cells from group H secretor subjects. In view of the high incidence of secretor individuals in the population, it would seem probable that the original donor of the HeLa cell strain was a secretor. However, our own searches for soluble H substance in tissue culture supernates from HeLa cell fractions has thus far been negative (13). It is possible that the antigenic composition of positively reacting HeLa cells is similar to that observed in group H buccal epithelium. If so, the mixed positive and negative clonal poulation might reflect a variant characterized by the loss of one, but not another immunodeterminant, and the variability of staining within clones could be related to the position of cells within the cell cycle. Such variability might be less apparent in the progeny of cells positive for the whole antigen complex if single immunodeterminants behaved differently with respect to metabolic phase. The relationship of blood group activity to metabolic phase in cultured cells has been described (4). An alternate interpretation is that single cells were not necessarily cloned and that the mixed composition of some clones represents a failure to transfer single cells. However, examination of suspensions prior to cloning indicated that about 90% were composed of single cells.

Negatively reacting cells would appear to represent H-less variants, a circumstance which has been described for other cell markers (7). Although remote, the possibility must be considered that these are not HeLa cells but cell contaminants devoid of blood group H. On the basis of a few karyotype studies, it could be shown that the chromosome complement was within the range expected for HeLa cells (14).

The distribution of fluorescence in positively reacting cells within clones was observed to be variable. This might reflect relocations of structural group H during the course of the cell cycle. It will be recalled that mixed agglutination of HeLa cells by anti-H was stronger in populations rich in mitotic cells than in those containing high proportions of interphase cells. Since the technique of mixed agglutination reflects only the presence of surface group H, an assessment of total synthesis may require alternate methods, particularly if intracellular blood group substance is present. We have examined populations of synchronized HeLa cells using fluorescent labeled anti-H reagent and have found different patterns of distribution between mitotic and interphase cells (13).

The potential value of blood groups as genetic markers has been commented upon (15), particularly in view of their stability in the intact organism. However, an alternate point of view may be necessary in comparing erythrocyte blood groups with those on nucleated cells in culture. Experiences with different cell markers have indicated degrees of instability whose origins are not always well defined, but are most likely related to the conditions imposed by tissue culture after separation from the parent organism (16). If immunological markers are utilized, the complexity of the immunodeterminants must be understood and defined if the reagents for their detection are to be accurate. Within the limitations imposed by these conditions, somatic cell markers, such as blood groups may be expected to illuminate mechanisms not as yet understood in mammalian cells, including the sequence of gene action and gene repression and derepression.

*Summary.* HeLa cells were separated into two portions by agglutination with anti-H

ulex reagent, and the unreactive supernatant cells (2) were studied in comparison with the original cell suspensions (1). Examination of clones derived from these populations using fluorescent labeled anti-H reagent indicated that a high percentage derived from (1) reacted positively. On the other hand, those derived from (2) consisted of negatively reacting or mixed positive and negative clones. Subclones from these populations behaved in a fashion which characterized the parent cell colonies.

*Note added in proof.* The findings of Bottomley, Trainer and Griffin, J. Cell Biol. 41:806:1969 call attention to the importance of HeLa strain differences, since chromosome and biochemical differences were found among seven strains tested. Possible associations between karyotype and group H reactivity are currently being sought in this laboratory.

1. Kelus, A., Gerner, B., and Coombs, R., Immunology **2**, 262 (1959).

2. Brand, G., Yunis, E., and Yunis, J., Nature **200**, 362 (1963).

3. Chessin, L., Bramson, S., Kuhns, W. J., and Hirschhorn, K., Blood **25**, 944 (1965).

4. Kuhns, W. J. and Bramson, S., Nature **219**, 938 (1968).

5. Masters, M., Kuempel, P., and Pardee, A., Biochem. Biophys. Res. Commun. **15**, 38 (1969).

6. Florey, L. L., Vox Sang. **11**, 137 (1966).

7. Fortelius, P., Saksela, E., and Saxen, E., Exp. Cell Res. **21**, 616 (1960).

8. Levintow, L. and Darnell, J. E., Jr., J. Biol. Chem. **235**, 70 (1960).

9. Boyd, W. and Shapleigh, E., J. Lab. Clin. Med. **44**, 235 (1954).

10. Kent, S., J. Histochem. Cytochem. **12**, 519 (1964).

11. Coons, A. and Kaplan, M. J., J. Exp. Med. **91**, 1 (1950).

12. Szulman, A., J. Exp. Med. **115**, 977 (1962).

13. Bramson, S. and Kuhns, W. J., Unpublished data.

14. Tjio, J. and Puck, T. T., J. Exp. Med. **108**, 259 (1958).

15. Race, R., and Sanger, R., "Blood Groups in Man," 5th Ed., Davis, Philadelphia, Pennsylvania (1968).

16. Merchant, D., and Neel, J. (Eds.) "Approaches to the Genetic Analysis of Mammalian Cells," Univ. of Michigan Press, Ann Arbor, Michigan (1962).

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