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# Immunological Characterization of HeLa Cell Subpopulations Enriched for Blood Group H or Its Precursor[1] (39923)

WILLIAM J. KUHNS,[2] GULTEKIN KANRA, AND CHARLOTTE PANN[3]

*New York University School of Medicine, Department of Pathology, New York, New York 10016, and Immunohematology Laboratory, Bellevue Hospital Center, New York, New York 10016*

Lectin fractions have been used by us to study the expression of blood group glycoconjugates on the surface membrane of cultured mammalian cells. HeLa cells and other epithelial cells with group H specificity exist as a mixed population for blood group H. A portion of these cells is H positive (1) and reacts with lectins specific for terminal L-fucose, the major immunodeterminant sugar of H antigen (2). Glycoconjugates within the remaining population of H-negative cells appear to function as acceptors for L-fucose when conversion occurs from H− to H+ at the time of cellular maturation (3). The penultimate acceptor sugars are D-galactose in $\beta$ linkage with *N*-acetylglucosamine, a disaccharide which in the 1-4 configuration occurs in common with structures other than blood groups. Pneumococcal type XIV polysaccharide is one of these materials. As a result antipneumococcal type XIV is reactive with the blood group acceptor disaccharide as well as with its corresponding antigen (2).

In the present studies, purified subpopulations positive or negative for the H antigen were derived from HeLa cells by velocity sedimentation on an albumin gradient by sensitizing H+ cells with purified anti-H ulex reagent, incubating this mixture, and removing the rosettes which formed from the remaining free cells. In this way a purified suspension of H− cells could be derived in the supernatant. Free H+ cells were derived from rosettes after red cells had been detached in the presence of L-fucose and removed by sedimentation on a gradient. Each subpopulation of HeLa cells was further characterized histologically by immunofluorescence techniques.

*Materials and methods. HeLa cell strain.* Strain HeLa S-3 was obtained from Dr. Rody Cox of the New York University School of Medicine and from Dr. Elliott Robbins of the Albert Einstein School of Medicine of Yeshiva University. The cells were maintained in continuous culture in our laboratory and were monitored for the presence of bacteria and PPLO.

*Nutrient medium.* Eagle's minimal essential medium (MEM) modified according to Levintow and Darnell (4) was employed for standard maintenance of the HeLa line. The medium contained a final concentration of 15% fetal calf serum, penicillin (100 units/ml), streptomycin (100 $\mu$g/ml), and 0.25 ml of fungizone (6.25 $\mu$g/ml). Whole medium utilized for these culture studies was assayed for the presence of soluble H antigen and for anti-H by inhibition and agglutination techniques (5, 6); neither substance was demonstrated. Monolayers were passaged at 5 to 7 days following brief treatment with 0.1% trypsin solution.

*Inocula and growth conditions.* The initial inoculum of $5 \times 10^5$ cells was implanted in small plastic dilution bottles. An increase in cell numbers to $2 \times 10^6$ occurred during exponential growth. Only cells in this phase were used.

*Disaggregation of monolayers.* Cell suspensions were derived from washed monolayers using treatment with 0.1% trypsin for 10 min. Cell counts were carried out, pools were prepared, and volumes were adjusted with phosphate-buffered saline (PBS) at pH 7.0 so that $10^7$ cells were contained in 0.5-ml aliquots.

*Lectin fractions and purified antisera.* High-titered anti-H ulex (anti-$H_F$) reagent was prepared from the seeds of the plant *Ulex europeus* for mixed agglutination studies. The crude extract was purified by pre-

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[2] Requests for reprints should be addressed to Dr. Kuhns, University of North Carolina School of Medicine, Department of Pathology, Chapel Hill, NC 27514.

[3] Deceased.



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cipitation at 70% saturation with ammonium sulfate and was gel filtered on Sephadex G-200 (7). The active fractions were reprecipitated with ammonium sulfate dissolved in 0.01 *M* phosphate buffer, pH 8.0, and placed on an ion-exchange column (DEAE-Sephadex A-50). A highly purified fraction was released upon treatment with 0.2 *M* phosphate buffer, pH 7.5; the agglutinating activity of this fraction against group O red cells was inhibitable specifically by L-fucose. The diluted lectin fraction which contained 10 $\mu$g of protein/ml and had a titer of 1:512 was utilized in experiments to obtain purified populations of H+ cells from H+/H− cell mixtures. Cells separated from such mixtures were 90 to 95% pure. Reagents for control studies were prepared by absorbing lectins with washed group O erythrocytes.

*Mixed agglutination.* Testing for blood group H was carried out by a modification of the mixed agglutination technique described by Kelus *et al.* (8). In controls, group AB erythrocytes were utilized in mixture with anti-H-sensitized cells.

*Fluorescent labeling technique.* A triple sandwich technique was utilized for the detection of H antigen (3, 9). Coverslip preparations of cells were fixed in 0.25% glutaraldehyde (3). High-titered ulex extract (anti-H) was added to the coverslip for 30 min at room temperature in a humidified environment, after which the cells were washed three times with PBS, 5 min for each wash. Rabbit anti-ulex reagent was added in the same manner and the mixture was washed. Fluorescein-labeled goat antirabbit $\gamma$-globulin (Microbiological Associates) was added to the coverslip and reacted 30–40 min at 37° in a humidified environment followed by washing three times in PBS. Negative control coverslips were stained with ulex reagent absorbed with group O red cells or ulex reagent neutralized with L-fucose. All coverslips were mounted in 10% buffered glycerol and examined under an AO fluorescent microscope using a heavy blue filter.

Staining of fixed coverslip preparations for blood group H precursor (anti-pneumococcal type XIV reactive) was as follows: Rabbit anti-pneumococcal type XIV serum provided by Dr. Michael Heidelberger was incubated with the fixed cells at 37° for 30 min and washed. Staining with fluorescent-labeled goat antirabbit globulin was carried out as described above.

*Cell separation technique.* Separation of free H− cells from an H+/H− cell mixture which had first been treated with ulex anti-$H_F$ and group O red cells was carried out in an albumin gradient at room temperature by a modification of the procedure described by Eden *et al.* (10). Figure 1 schematizes the apparatus utilized for velocity sedimentation. An albumin gradient was introduced into a wide siliconized glass sedimentation funnel at controlled speed with a variable-speed pump. The following preparatory procedures were carried out. The gradient maker was filled with 100 ml of MEM-1% bovine serum albumin (BSA) mixture, and the connecting tube between the sedimentation funnel and the pump was clamped. Four to five milliliters of 30% BSA in the bottom of the funnel were layered first with 15 ml of MEM and then with 15 ml of cell mixture containing 20 × $10^6$ HeLa cells and 150 × $10^6$ human group O erythrocytes. Thereafter, MEM-BSA mixture from the gradient maker was introduced into the sedimentation funnel at the rate of 300 ml/hr. Under these conditions H+ HeLa cell group O red cell rosettes (Fig. 2) sedimented toward the bottom of the funnel and free H− cells remained close to the top. The efficacy of the separation was monitored microscopically beginning at 15 min, and quantitative removals of free cells 85 to 90% pure could be made at 10 to 15 min thereafter (Table I).

*Separation of free H+ HeLa cells from rosettes.* H-antigen-negative HeLa cells were separated away from sedimented rosettes which contained H+ HeLa cells. One milliliter of a 0.2 *M* solution of L-fucose was added to the cell button which was then agitated gently to resuspend the rosettes. Agitation was continued and the suspensions were monitored microscopically until most of the adherent group O red blood cells were freed from HeLa cells. Gradient sedimentation was repeated, and the supernatant HeLa cell suspension was removed from the bottom pellet of red cells and washed with PBS. This sedimentation and washing technique was also utilized to

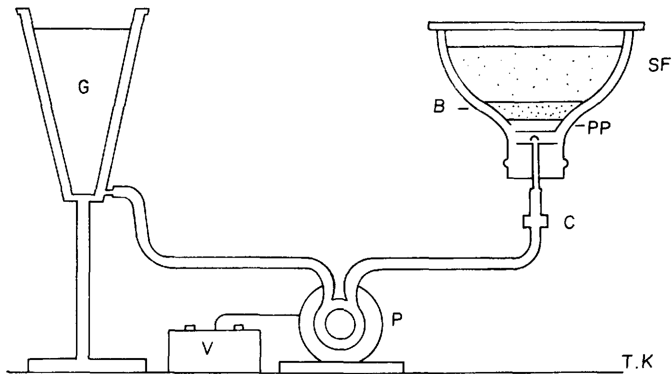


FIG. 1. Schematic representation of the apparatus used for cell separation. (G) gradient maker; (V) variable speed controller; (P) pump; (PP) porous polyethylene; (C) clamp; (B) bovine albumin; (SF) sedimentation funnel.

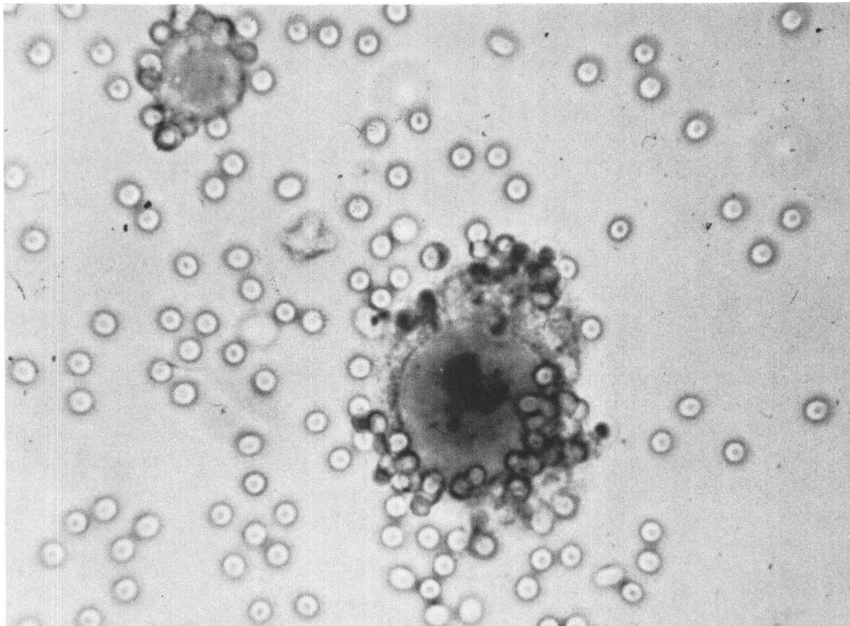

FIG. 2. Rosettes comprised of HeLa H-antigen-positive cells, sensitized with anti-$H_F$ ulex reagent, surrounded by adherent human group O erythrocytes. ×360.

prepare free HeLa H− cells which had been admixed with erythrocytes in the original suspension.

*Results. Cell separation.* The results of a typical separation experiment are shown in Tables I and II. The initial quantity of HeLa cells numbered 20 × $10^6$. Following the addition of ulex anti-$H_F$ reagent and human group O erythrocytes the count of HeLa H+/red cell rosettes was found to be 5.6 × $10^6$, and thus the percentage of H+ cells in the mixture was calculated at 28% of the original cell count (Table II). The recovery of HeLa H+ cells in the sedimented and L-fucose-treated population was calculated to be 2.3 × $10^6$, or approximately 40% of H+ cells contained in rosettes in the initial mixture (Table II). HeLa H− cells (11.8 × $10^6$, 59% of the original HeLa cell count) admixed with free erythrocytes and a small percentage of rosettes accounting for 5-10% of recovered cells were obtained in the original supernatant. H− cells (8 × $10^6$; 40% of total HeLa cells) were re-

TABLE I. PURIFICATION OF HeLa H-ANTIGEN-NEGATIVE CELLS FROM H+/H− CELL MIXTURE BY VELOCITY SEDIMENTATION ON AN ALBUMIN GRADIENT

| | Number of cells at start | | Recovery of supernatant HeLa cell subpopulation | | | |
|---|---|---|---|---|---|---|
| Lectin used for separation | Group O erythrocytes for separating sensitized H+ cells as rosettes (× $10^6$) | HeLa cells (× $10^6$) | Type | Number (× $10^6$) | Percentage of original | Purity |
| Anti-H ulex | 150 | 20.0 | H− | 11.8 | 59 | 90–95 |
| None<br>Gradient separation for red cell removal | | | | 8.0 | 40 | |

TABLE II. RECOVERY OF HeLa H-ANTIGEN-POSITIVE CELLS FROM ROSETTES FOLLOWING SEPARATION FROM H+/H− CELL MIXTURE ON AN ALBUMIN GRADIENT

| | |
|---|---|
| Composition of initial mixture | |
| Total anti-H-sensitized HeLa cells (× $10^6$) | 20.0 |
| Total group O erythrocytes (× $10^6$) | 150 |
| Number of HeLa H+ cells contained in rosettes (× $10^6$) | 5.6 |
| Percentage of rosettes | 28 |
| Composition of sedimented population | |
| Total HeLa cells (× $10^6$) | 3.6 |
| Total free HeLa H+ cells following L-fucose treatment | 2.3 |
| Percentage of recovery from rosettes | 40 |
| Properties of recovered free H+ HeLa cells | |
| Reactive upon fluorescent labeling with specific reagents | |
| Rosettes found following treatment with anti-H ulex and group O erythrocytes | |

covered in the red cell-depleted supernatant (Table I), of which approximately 90% could be considered H− when retested by the techniques of mixed agglutination and immunofluorescence.

Purified suspensions of both types of cells were characterized using fluorescent labeling techniques. In the case of separated H− cell suspensions which were fixed and stained, reactivities were stronger when rabbit anti-pneumococcal type XIV and fluorescent-labeled goat anti-rabbit sera were used as reagents than in fixed H− cell preparations when the anti-H ulex, rabbit anti-ulex, and fluorescent goat anti-rabbit sandwich technique was employed (Figs. 3 and 4). Reciprocal results were noted when separated and fixed H+ cells were similarly tested (Figs. 5 and 6).

The separated H+ cells, upon retesting by the mixed agglutination technique, retained the ability to form rosettes in the presence of specific anti-H and group O erythrocytes. This finding, coupled with the demonstration of specific H-antigen activity by fluorescent labeling indicated that group H sites were not irreversibly modified following limited exposure to anti-H ulex extract.

*Discussion.* The pattern observed when purified HeLa H-positive and H-negative cells were characterized by immunofluorescence suggested that most cells within the former population possessed an abundance of H-antigen-reactive sites, whereas only small areas of precursor (anti-pneumococcal type XIV reactive) appeared to be present on some cells. In contrast, the relatively low degree of reactivity of H-negative cells to anti-$H_F$ reagent suggested a paucity of these sites, but on the other hand diffuse areas were reactive against anti-pneumococcal type XIV and were presumed to represent H-antigen precursor.

The technique of cell separation using antigen marker-specific lectins is important as an approach to the procurement of highly purified single populations whose membrane components can then be characterized (11). Little apparent damage was caused to specific receptors in H+ cells following reversible combination of anti-H ulex with cellular H antigen as judged by the occurrence of positive reactions when specific immunofluorescent staining was carried out. A more complete assessment may be possible if a system can be provided for selectively removing H− cells from H+

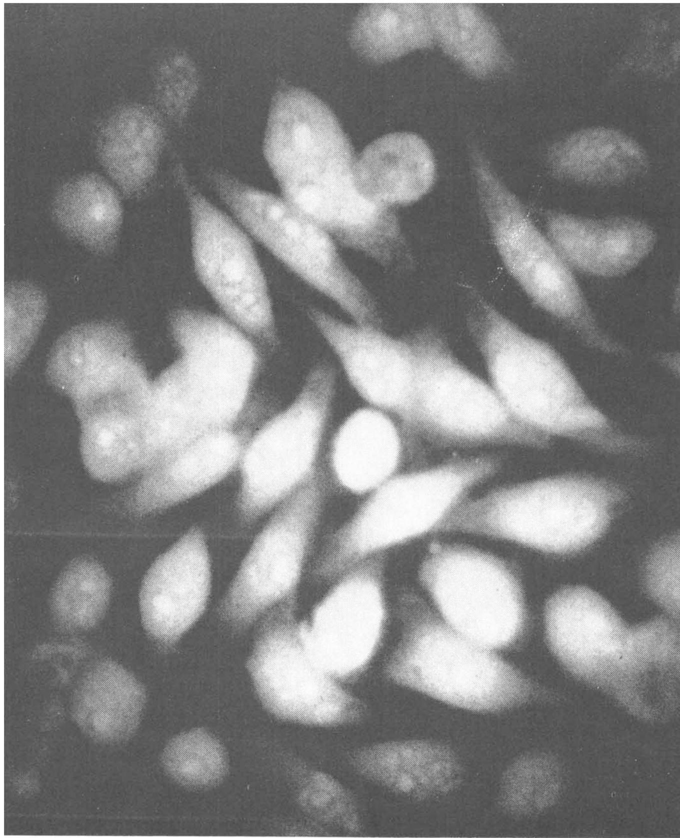

FIG. 3. Coverslip preparation of purified glutaraldehyde-fixed HeLa H-antigen-negative cells tested for precursor substance: Cells were treated with rabbit anti-pneumococcal type XIV, washed, and stained with fluorescent-labeled goat anti-rabbit serum. Strong fluorescent staining pattern is noted in most cells. ×400.

cells, in other words, the reciprocal of the present experiment. The feasibility of utilizing anti-pneumococcal type XIV in combination with antigenically appropriate erythrocytes for sedimentation experiments is under study.

Earlier findings of ours have indicated that the H antigen is growth and cell cycle dependent (3, 12) and that antigenic competence (i.e., phenotypic expression) is acquired as epithelial or epitheloid cells divide and differentiate. This raises the question whether undifferentiated cell populations are mixed with respect to the presence or absence of precursor (type XIV antibody reactive) antigen. Immunoselection experiments such as described above may be of importance in resolving such questions; they may also be useful in providing uniform populations for assaying specific glycosyl transferase enzymes, the direct gene products necessary for expression of these blood groups.

It is commonly believed that purified soluble blood group substance is chemically heterogeneous, apparently the result of imperfect or incomplete oligosaccharide chain formation (2, 13, 14). This in turn would appear to stem from the imperfect behavior of specific transferase enzymes which catalyze addition of single sugars from nucleotides to growing oligosaccharide chains (14). Similar forms of heterogeneity are thought to occur on cells which express blood groups (15, 16). We are augmenting the present studies using fixed, paraffin-embedded, sectioned, and stained HeLa cells in order to carry out replicate staining of adjacent sections of single cells for H antigen and its precursor. Preliminary ex-

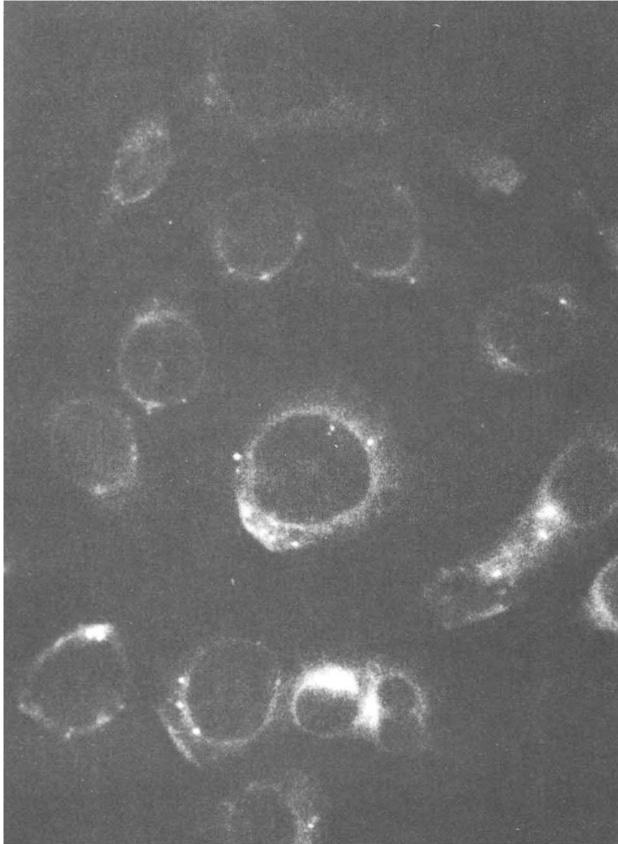

FIG. 4. Purified glutaraldehyde-fixed HeLa H-antigen-negative cells treated with anti-$H_F$ ulex reagent, rabbit anti-ulex, and fluorescent-labeled goat anti-rabbit serum. Many cells stain negatively, and some show small patches of fluorescent staining. ×400.

periments indicate that both substances coexist on single cells, but differ in their degree of individual expression depending upon the state of cellular differentiation. These findings will be reported separately.

Cancer cells *in vivo* exist as part of a mixed cell population. Methodologies which can characterize normal and abnormal components of these populations with reasonable accuracy should yield important clues to the innate behavior of cancer cells and should also prove useful in cancer diagnosis or prognosis. Some of these techniques are immunological in nature based upon changes from the normal in the antigenic composition of tumor cells (11). The ABO(H) blood group antigens are potentially important in this regard since (a) they are chemically defined immunodeterminants whose genetic structure and biosynthesis are reasonably well understood; (b) the serological techniques used for their characterization on red blood cells can be adapted for studies of blood groups on tissues and organs, or cells in culture, and can also be employed for separation of components of mixed cell populations; and (c) ABO(H) blood group antigen deficiencies or losses have been reported in carcinoma cells in comparison with their normal cellular counterparts (17, 18). The availability of cell purification methods should make possible more intense study of these and other cancer-induced membrane defects.

*Summary.* Purified HeLa cell subpopulations positive or negative for the H blood group antigen were obtained from H-positive/H-negative mixtures using velocity cell separation on an albumin gradient. The

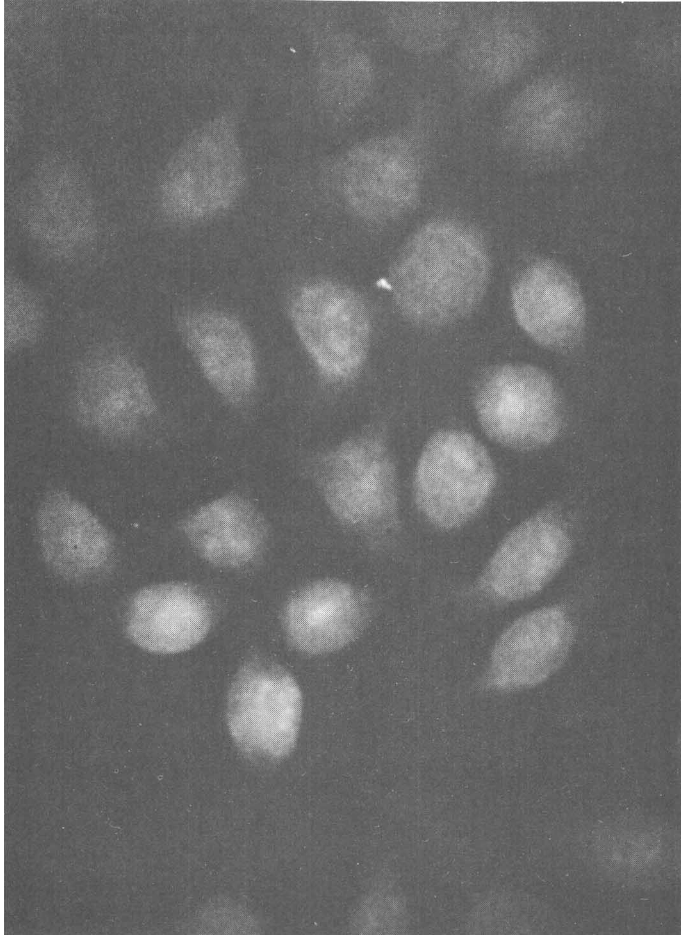

FIG. 5. Coverslip preparation of purified glutaraldehyde-fixed H-antigen-positive cells tested for precursor substance using technique described in Fig. 3. Fluorescence is weak or absent. ×400.

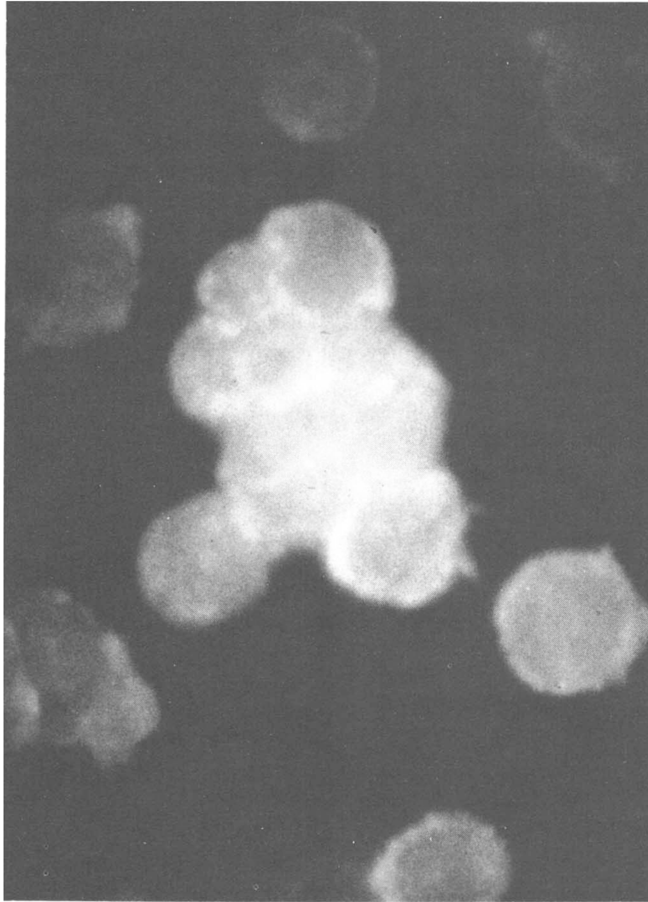

FIG. 6. Purified glutaraldehyde-fixed HeLa H-antigen-positive cells tested for H antigen using technique described in Fig. 4. Strong fluorescence is noted in many cells, punctate or weak to moderate fluorescence in some others. ×400.

identity of H antigen or its anti-pneumococcal type XIV reactive precursor was demonstrated on fixed cells by fluorescent labeling experiments. H-negative cells reacted better when treated with rabbit anti-pneumococcal type XIV antibodies and fluorescein-labeled anti-rabbit serum than when tested for H antigen using a triple sandwich method. Reciprocal findings were demonstrated with H-positive cells.

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