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LEPTOTRICHIA BUCCALIS HEMAGGLUTINATION IN CELL BINDING AND SALI--ETC(U)
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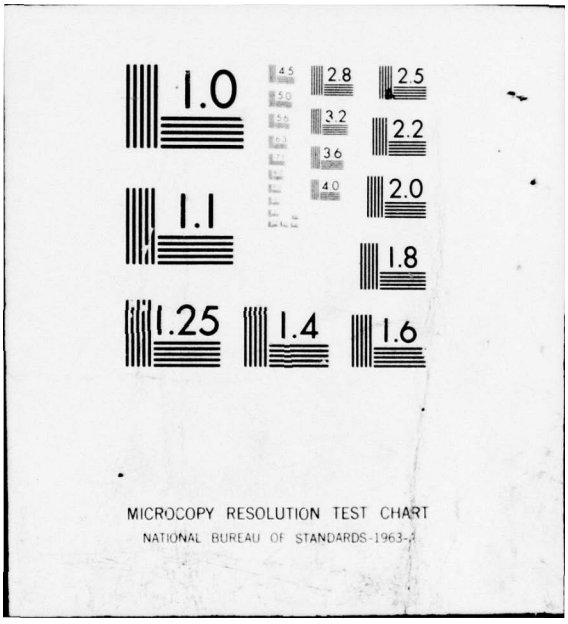
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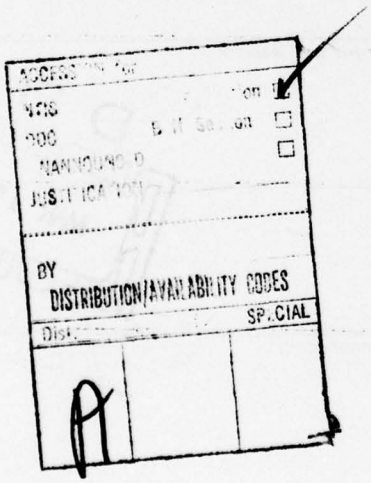

Leptotrichia buccalis Hemagglutination in Cell Binding and Salivary Inhibition Studies

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Abstract

The characteristic hemagglutination (HA) of *Leptotrichia buccalis* was used for measuring its attachment to various human cells and for determining if saliva contained hemagglutination inhibition (HI) factors. The microbial strain utilized displayed the characteristic EM morphology of *L. buccalis*. Sonicated preparations of the organism were tested for HA activity before and after adsorption with human cells. Buccal epithelial cells, RBC, HeLa and embryonic kidney cells all bound the HA fragments of the organisms. The bacterial fragments on the cells could be observed by fluorescent antibody testing. The fragments were released from the cells used for adsorption with chelators and, upon addition of $CaCl_2$, the HA activity returned. Whole saliva displayed hemagglutination inhibition activity in a manner suggesting a binding site interaction. The similarity of the HA activity of *L. buccalis* and *F. nucleatum* are discussed as are the relationships of cell binding to colonization of the organisms and immunopathology to host cells.

Introduction

Leptotrichia buccalis has been observed to cause hemagglutination (HA) of a variety of mammalian red blood cells (Kondo, Sato, and Ozawa, 1976 and Falkler and Hawley, 1977a). The genus *Leptotrichia* is placed in the family *Bacteroidaceae* along with another genus *Fusobacterium*, oral strains of which have demonstrated HA activity (Falkler and Hawley, 1977b). The HA activities may be important as a mechanism for studying microbial cell attachment to epithelial surface and microbial cell to cell attachment. Such mechanisms may be important in colonization of the organisms in the gingival crevice and the binding of antigenic fragments of the organisms to human cells could allow immunopathology to occur.

As receptors on epithelial cells may demonstrate blood group reactive substances and mucinous glycoproteins may mimic these receptors (Williams and Gibbons, 1975), experiments were undertaken to see if the *L. buccalis* HA active fragments could bind to buccal epithelial cells, red blood cells, and other human cell lines. Saliva was studied to determine if there were factors present which would inhibit the HA activity of these organisms.

Materials and Methods

Growth of organism and HA preparation.

L. buccalis (ATCC #19616) and *Fusobacterium polymorphum* (ATCC #10953) now considered to be *F. nucleatum* (Moore & Holdeman, 1974) were grown using the BBL gas pak anaerobe jar system. After 18 h the organisms were harvested by centrifugation at 10,000 x g for 10 min and washed 3 times in 0.01 M phosphate buffered 0.15 M saline, pH 7.2, containing 0.02% sodium azide, designated PBS. Organisms taken directly from culture and after washing were used for electron microscopic observation. Other washed

organisms were resuspended in PBS to 10% or 30% suspensions. The 10% suspensions were used directly for HA activity testing. The 30% suspensions were sonicated in a dry ice alcohol bath using a Branson sonicator with a probe tip (6 amps, 9, 20 sec bursts). The sonicated preparation was then centrifuged at 10,000 x g, the supernatant fluid being referred to as LB 10,000, the sediment was resuspended in an equal volume of PBS and centrifuged at 2,000 x g for 10 min, the resulting supernatant fluid designated LB 2,000. The remaining sediment was treated according to the method of Garcia, Alexander and Kay (1975) for the preparation of cell walls.

HA and Hemagglutination Inhibition (HI) Testing.

The HA and HI tests were modifications (Falkler and Hawley, 1977b) of the microtiter tests described by Crawford *et al.* (1971). The HA activity of 10% suspensions of whole *L. buccalis* cells, LB 10,000, LB 2,000, LB cell walls, and the LB 10,000 HA preparations after adsorption with a variety of human cells was tested. The HI activity of human saliva (unstimulated saliva collected from 10 individuals, diluted 1:4 with PBS pH 7.2 and centrifuged at 600 x g for 10 min), rabbit antisera to *L. buccalis* and *F. nucleatum*, normal rabbit sera, and gelatin at 50 mg/ml was determined. Checkerboard dual titrations of *L. buccalis* HA preparations versus saliva dilutions were also performed.

Preparation of Rabbit Antisera.

Antisera were prepared against *L. buccalis* and *F. nucleatum* as previously described (Falkler and Hawley, 1977b) using as antigen preparations the supernatant fluids resulting from centrifugation of sonicated preparations of the organisms at 10,000 x g for 10 min.

Electron Microscopy.

L. buccalis and *F. nucleatum* were fixed with ruthenium red and osmium tetroxide according to the methods of Cagle, Pfister, and Vela, (1972). The

fixed cells were then dehydrated in graded ethanol and embedded in Spurr low viscosity embedding media (Spurr, 1969). Thin sections were cut and stained with saturated uranyl acetate in 50% ethanol for 20 min followed by lead citrate, pH 12, for 5 min. The sections were examined in a previously calibrated, astigmatic, and aligned Siemens Elmiskop 1A electron microscope at an accelerating voltage of 80 kv.

Absorption of LB 10,000 with Human Cells.

Human red blood cells (types A+, A-, B+, AB+, and 0+) in Alsever's solution were washed 3 times in PBS, with centrifugation between washes at 400 x g for 10 min. Human buccal epithelial cells were scraped from the oral buccal surfaces of 10 individuals using wooden applicator sticks and the collected cells washed 3 times in PBS. HeLa cells and human embryonic kidney cells were grown in MEM plus 50 µg/ml gentamicin in glass 32 oz. prescription bottles at 37 C in a 5% CO_2 environment. Complete monolayers were removed by dislodging with a rubber policeman. The harvested cells were also washed 3 times with PBS. Forty per cent suspensions (packed volume after centrifugation at 500 x g for 10 min diluted 1:2.5) of the human cells were incubated with an equal volume of LB 10,000 at 37 C for 30 min followed by centrifugation of the cells at 400 x g for 10 min. Controls included 1:1.6 dilutions of the LB 10,000 with PBS. The supernatants and controls were then tested for HA activity in the standard test with SRBC. The sedimented cells were then washed twice with PBS and resuspended in PBS to a 20% suspension. The suspensions were utilized for the preparations of slides for fluorescent antibody testing. Aliquots (0.5 ml) were treated with an equal volume of 0.5 mM EDTA, 1.0 mM EDTA, or 0.85% saline for 15 min at 37 C and then centrifuged at 400 x g for 10 min. To the resulting supernatants was then added 0.5 ml of 0.5 mM $CaCl_2$ and after incubation at room temperature for 15 min, the HA activities were determined.

Fluorescent Antibody Testing.

Twenty percent suspensions in PBS of oral epithelial cells, HeLa cells and

human embryonic kidney cells before and after adsorption of the LB 10,000 preparation were used for fluorescent antibody testing. The suspensions were allowed to air dry on the slides and then fixed for 1 min in cold acetone. The indirect fluorescent staining procedure followed the method in Weir (1969) using rabbit anti-*L. buccalis* sera (1:4) followed by fluorescein labeled goat anti-rabbit sera (Miles) diluted 1:8 and counterstained with 0.2% Evan's blue. Controls included the use of cells not exposed to LB 10,000, normal rabbit sera or saline instead of anti-LB sera,and fluorescein labeled goat anti-rabbit sera without prior exposure of the cells to anti-LB sera.

Results

The electron microscopic studies verified the *L. buccalis* organisms used in these experiments had the following structural characteristics: external hair-like projections, a gram-negative cell wall, and the presence of division septae (Fig. 1). Neither the hair-like projections or division septae could be observed in similar preparations of *F. nucleatum*.

The washed LB organisms in a 10% suspension displayed a HA titer of 1:512. The LB 10,000 preparation (880 µg protein/ml) and the cell wall preparation (1 mg/ml) showed a 1:128 and 1:32 HA activity respectively.

As can be seen in Table 1, adsorption of the LB 10,000 preparation (one HA unit = 1:128) with human RBC, oral epithelial cells, HeLa and human embryonic kidney cells removed HA activity 2 to 4 fold. The greatest absorption was observed with the HeLa cells (4 fold removal), the least being with the kidney cells (2 fold removal). The adsorbed cells were washed with PBS and then treated with 0.5 or 1 mm EDTA for release of the HA active moiety from the cells. The HeLa and oral epithelial cell supernatant fluid displayed a 1:4 and 1:2 HA activity respectfully with both concentrations of

chelator and no HA activity could be detected in the supernatant fluid from the EDTA treated embryonic kidney cells.

The LB 10,000 adsorbed cells were positive for the presence of LB antigen by the use of the indirect fluorescent antibody technique. Oral epithelial cells (Fig. 2), HeLa cells (Fig. 3) and human embryonic kidney cells (Fig. 4) all displayed fluorescence. The controls for this technique were negative.

Table 1. Absorption of LB 10,000 with Human Cells[a]

Cell Source	Titer after Absorption
Oral Epithelial	1:16[b]
HeLa	1:8
Embryonic Kidney	1:32
RBC type AB+	1:16
" type A-	1:16
" type O+	1:16
" type A+	1:16
" type B+	1:16
No adsorption (1:16)	1:128

[a]40% suspensions
[b]titer displaying at least 2+ HA activity

Hemagglutination testing with 2 HA units of LB 10,000 (1:64) and ten unstimulated saliva samples displayed varying degrees of inhibition (Table 2). Titers ranged from 1:4 to 1:1,024. HI testing was also done on samples where the saliva dilutions were dual titrated against various LB 10,000 dilutions. In these experiments the inhibition activity was shown to be dependent upon the LB 10,000 concentration. Inhibition increased with LB

dilution until maximum inhibition was observed at a 1:128 dilution of LB 10,000 (Table 3). Antisera prepared to *L. buccalis* also inhibited the LB 10,000 HA activity (1:128 and 1:64 for rabbits A and B respectively) however antisera to *F. nucleatum*, normal rabbit sera, commercial rabbit sera, and gelatin at a concentration of 50 mg/ml had no HI activity. The anti-*L. buccalis* sera have shown titers (approx. 1:1,024) with homologous antigen preparations in indirect passive HA testing with tanned SRBC.

Table 2. HI testing of human saliva with LB 10,000[a]

Saliva sample designation	HI titer
A	1:4[b]
B	1:64
C	1:64
D	1:64
E	1:64
F	1:128
G	1:128
H	1:256
I	1:512
J	1:1024

[a] 2 HA units = 1:64

[b] dilution showing at least a 2+ HI

Table 3. HI Titers Resulting From Dual Titrations of Saliva Samples With HA Preparation.

HA[a] Preparation Dilutions	Titer[b] of Saliva I	J
1:4	8	8
1:8	32	16
1:16	32	32
1:32	64	64
1:64	1024	512
1:128	1024	1024

[a]LB 10,000 (2 HA units = 1:64)

[b]reciprocal of titer displaying 2+ HI

Discussion

As only one *L. buccalis* strain was used in this study we deemed it important to document the presence of this particular organism. Electron-microscopic studies have shown *L. buccalis* to have characteristic features so this approach was taken. The characteristic morphology reported for this organism by Listgarten and Lai (1975) and Kondo, Sato, and Ozawa (1976) was observed.

It appears that the HA activity of *L. buccalis* and *F. nucleatum* is via similar or identical mechanisms. In both instances a heat labile component of the outer surface of the microorganisms is involved (Kondo, Sato, and Ozawa, 1976 and Falkler and Hawley, 1977b). Kondo, Sato, and Ozawa (1976) suggested that the HA activity was associated with the characteristic hair-like surface

structures seen on the outer surface of *L. buccalis*. We haven't observed these on the surface of *F. nucleatum* however the HA active moiety of *F. nucleatum* like that of *L. buccalis* is very heat labile. It is possible that similar types of HA moieties could present themselves in different structures in the two genera.

The HA activity of both organisms and the human cell binding activity requires the presence of Ca^{++} as demonstrated in experiments using chelators. The HA activities of both have been inhibited by sugars containing galactose as the terminal monosaccharide (Kondo, Sato, and Ozawa, 1976 and Falkler, unpublished observations). Galactose is a major carbohydrate of red blood cell membranes and it has been shown that blood group determinants can be observed on other human cells (Winzler, 1970 and Williams and Gibbons, 1975). Although these organisms are relatively asaccharolytic one could hypothesize that they may have a galactose binding protein on their outer surface which allows this binding activity. This may be an adaptation by the organisms as a colonization mechanism to attach to cells bearing surface galactose residues.

The immunologic basis of periodontal diseases is antigens of microorganisms gaining entrance to the gingival tissues and upon reaction with antibody, T lymphocytes and effector systems resulting in immunopathology. This study demonstrates that antigenic fragments of *L. buccalis* can attach to a variety of human cell types. As a result of immunologic reaction, the cells to which these fragments are attached may be damaged. Studies involving the attachment of members of the family *Bacteroidaceae* to gingival fibroblasts and neutrophiles are in progress.

It is of interest that rabbit antisera prepared to *L. buccalis* and *F. nucleatum* displayed HI activity with specificity. Anti-*L. buccalis* sera

only displayed HI activity with *L. buccalis* HA preparations, while anti-*F. nucleatum* sera showed no inhibitory activity. In reported studies we have shown just the opposite with anti-*F. nucleatum* sera showing inhibition only with *F. nucleatum* HA, anti-*L. buccalis* sera having no effect (Falkler and Hawley, 1977b). In the light of the apparent similar HA mechanisms by the two organisms, the specificity of this reaction can be explained if the HA active moieties are not the antigenic determinants but if they are located close enough to the antigenic determinants so that the presence of antibody on a determinant neighboring the HA active moiety would sterically inhibit the HA moiety's binding to the red blood cell receptor.

Factors were present in whole saliva which inhibited the HA activity of *L. buccalis*. Kondo, Sato, and Ozawa (1976) reported that saliva allowed the binding of *L. buccalis* to enamel powder and caused aggregation of the organisms. A specific binding interaction was suggested in this study as the dual titration revealed the HI activity of the saliva was dependent upon the concentration of the *L. buccalis* HA preparation. If the inhibition did not involve binding sites it would have occurred at all HA preparation concentrations.

Salivary glycoproteins have been observed to bind to oral streptococci (Williams and Gibbons, 1975) and to plaque coccobacilli (Hay, Gibbons and Spinell, 1971). Also IgA has been reported to play a role in adherence by oral microorganisms (Williams and Gibbons, 1972). Currently studies in this laboratory are being undertaken to determine if the inhibitor in saliva is a non-antibody glycoprotein or specific antibody. In either case it is suggested that the salivary inhibition of HA may afford an anti-colonization mechanism or a means of preventing antigenic fragments of the organisms from binding to cells of the host.

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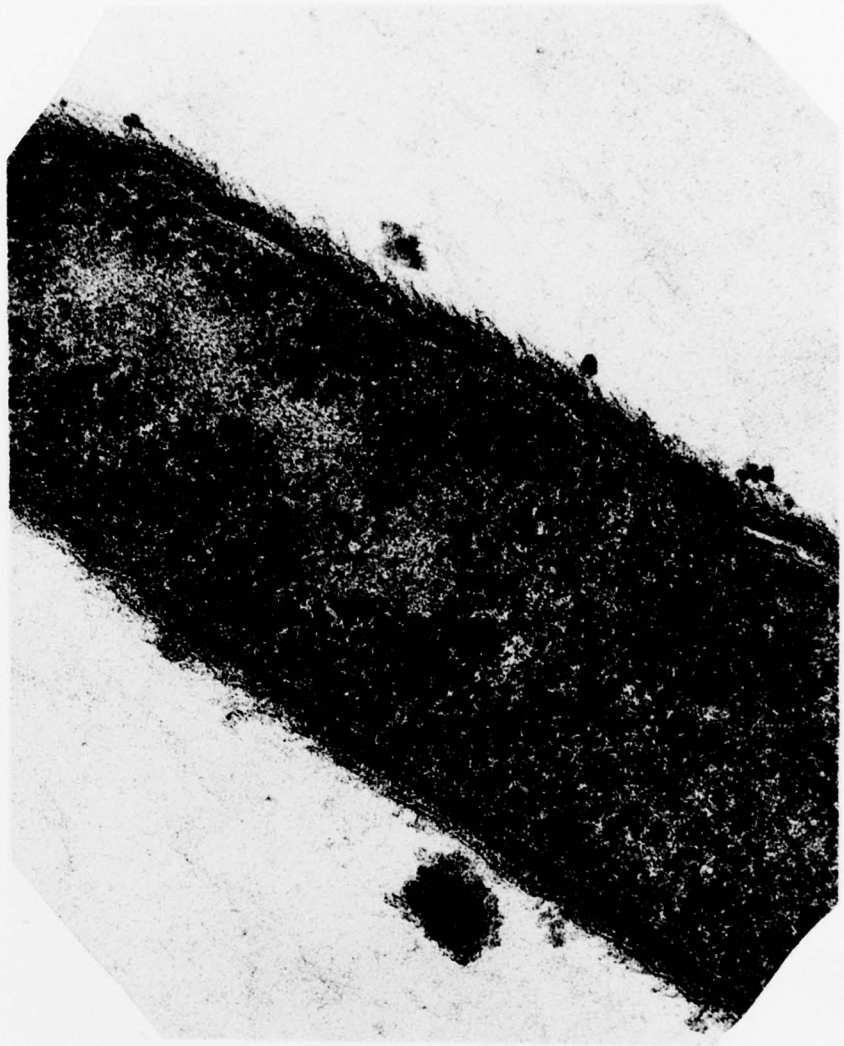
Figure Captions

Fig. 1. Electron micrograph of a section of *L. buccalis* ATCC #19616 showing a nap of short hair-like surface projections (H) and division septae(S). X141,000

Fig. 2. Fluorescence of adsorbed oral epithelial cells stained with rabbit anti-*L. buccalis* sera and fluorescein labeled goat anti-rabbit globulin. X400.

Fig. 3. Fluorescence of adsorbed HeLa cells stained as in Fig. 2. X400.

Fig. 4. Fluorescence of adsorbed human embryonic kidney cells stained as in Fig. 2. X400



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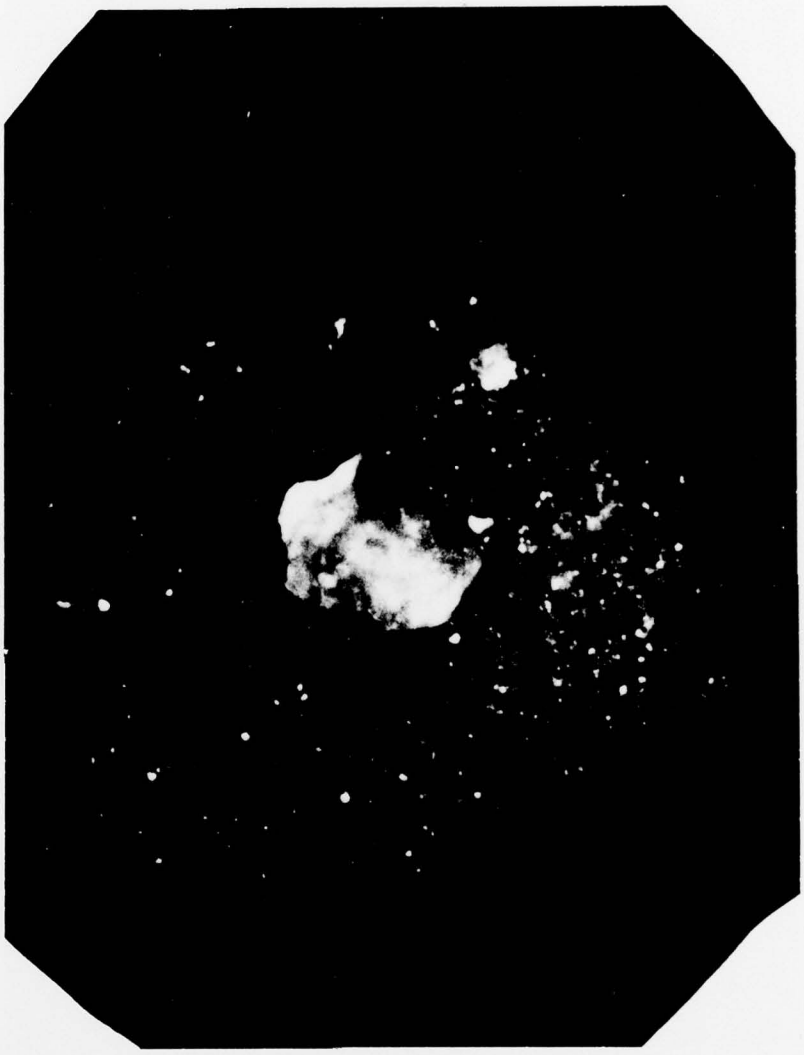

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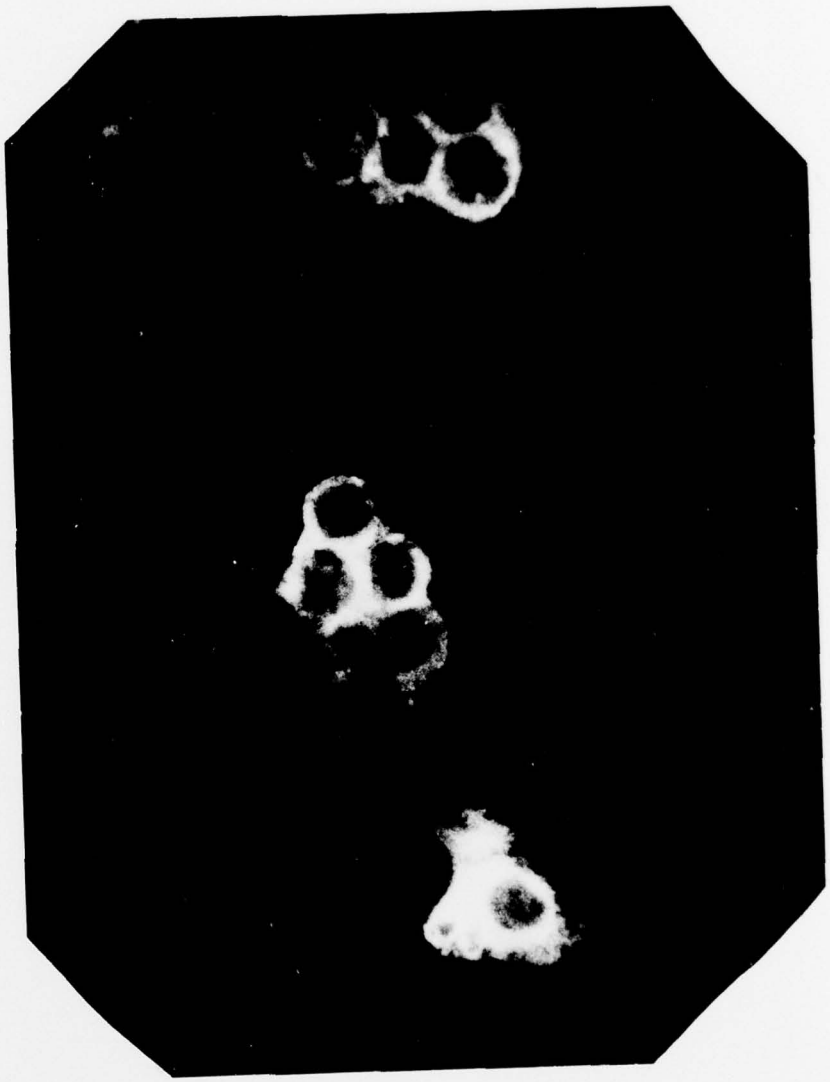

FIG 3

FIG 4.