

# Effect of Human Serum and Some of Its Components on Neutrophil Adherence and Migration across an Epithelium

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**Abstract.** The effect of human serum and some of its components on the process of transepithelial migration of human neutrophils was investigated in an in vitro system. 10% autologous serum caused an increase in neutrophil adherence to and migration across canine kidney epithelial cells. This increase in neutrophil binding also occurred if the epithelium but not the neutrophils had been preincubated with serum. The binding was lost if the serum was either preabsorbed over the kidney epithelium before use or heat inactivated. Indirect immunofluorescence studies indicated that IgG, IgM, and a component of C3 bound to the epithelial surface, whereas IgA, IgE, or C5a were not detectable. The majority of epithelial cells were immunofluorescent, however epithelial cells with varying degrees of reactivity were also apparent and ~5% of the epithelial cells did not bind IgG, IgM, and C3. When epithelia were simultaneously tested for the presence of either IgG, IgM, or C3, and bound neutrophils the few epithelial cells which did not bind IgG or IgM also did not bind C3 or neutrophils. Stud-

ies with monoclonal antibodies against Fc and C3 receptors indicate that neutrophil adherence to the epithelial surface was mediated predominately by the receptors for C3b and C3bi. In response to a chemotactic gradient, bound neutrophils were able to detach and migrate across the epithelium. A separate heat-stable factor(s) in serum was able to increase neutrophil migration across the epithelial monolayer. This factor acted independently of the factors which caused the increase in neutrophil binding as the increase in neutrophil migration also occurred under conditions (preabsorption over the kidney epithelium or heat inactivation) that prevented the increase in neutrophil binding. The increase in neutrophil migration may be caused by the permeability-increasing properties of this factor as both serum and heat-inactivated serum lowered the transepithelial electrical resistance an average of 38 and 35%, respectively, in 40 min. Upon removal of serum or heat-inactivated serum, the resistance returned 100 and 81%, respectively, in 5 h.

**N**EUTROPHILS must be able to adhere to and traverse the endothelium lining blood vessels (14) and, frequently, must be able to migrate across a second epithelium (24, 27, 30) in order to accumulate at inflammatory sites. In fact, neutrophils when stimulated appear capable of traversing every epithelium (26) regardless of its permeability (5, 10). The control of epithelial permeability is due to a specialized junction called the zonula occludens (tight or occluding junction), which appears ultrastructurally as a region of fusion between the outer leaflets of the plasma membrane of two adjacent epithelial cells (11, 25, 28). To study how neutrophils are able to migrate across such barriers we have developed an in vitro model system (8) which involves growing a confluent, polarized monolayer of Madin-Darby canine kidney epithelial (MDCK)<sup>1</sup> cells on micropore filters

(23). These cells form zonula occludens and develop a transepithelial electrical resistance, a measure of epithelial permeability, as high as 500 ohms/cm<sup>2</sup>. Neutrophils placed under conditions of chemotaxis readily traverse epithelia of low transepithelial electrical resistance but as the resistance increases the number of neutrophils traversing the monolayer decreases (7, 22). In vivo, inflammatory stimuli cause neutrophils to traverse epithelium with transepithelial electrical resistances similar to as well as significantly higher than that recorded in our in vitro system (1, 5, 10). This would suggest that other factors such as those found in serum and connective tissue, especially under pathological conditions, may participate in the process. The heterologous system we used (human neutrophils and serum, canine kidney epithelium) provided a means to study various serum factors, in particular, immunoglobulins, and complement on neutrophil adherence to and

1. *Abbreviations used in this paper:* Gey's, Gey's balanced salt solution containing 2% (wt/vol) BSA, penicillin (63 U/ml), and streptomycin (138 µg/ml); HIS, heat-activated serum; MDCK cells, Madin-Darby canine kidney epithelial

cells; serum, fresh human serum; ZASHIS, zymosan-activated serum which has been heat inactivated for 30 min at 56°C.

migration across an epithelium. Under the conditions in this study, IgG and IgM in the human serum bound to the apical epithelial surface, fixed complement, but did not lyse the epithelial cells. This resulted in a significant increase in neutrophil adherence to the epithelium which appears to be due primarily to neutrophil binding to C3 deposited on the epithelial surface. In addition, there was a significant increase in neutrophil migration across the epithelium which appears to be caused by a separate heat-stable component in serum which increased epithelial permeability.

## Materials and Methods

### Preparation of MDCK Monolayer

MDCK originally isolated from an adult, female dog kidney (provided by J. Leighton, Medical College of Pennsylvania, Philadelphia, PA) were maintained in culture by serial passage in Eagle's minimal essential medium with Earle's balanced salt solution, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% (vol/vol) fetal calf serum (CMEM) or were stored in liquid nitrogen after the addition of 10% (vol/vol) dimethyl sulfoxide to the culture medium. The cells were harvested with 0.25% trypsin-2 mM EDTA in Dulbecco's phosphate-buffered saline (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS), plated ( $1-2 \times 10^5$  cells/ml) on 13-mm-diameter micropore filters (cellulose acetate and nitrate, 0.45-µm pore; Millipore/Continental Water Systems, Bedford, MA) according to Misfeldt et al. (23) and grown in a 95% air/5% CO<sub>2</sub> atmosphere for 4-6 d at 37°C. Falcon tissue culture plasticware was purchased from J. & H. Berge, Inc. (Plainfield, NJ); serum and minimal essential medium were from GIBCO (Grand Island, NY).

### Transepithelial Electrical Resistance Studies

Transepithelial electrical resistance of MDCK monolayers grown on the micropore filters (3, 23) was determined before all experiments. The filters, used for chemotactic studies, were mounted between the two halves of a Ussing chamber (exposing 0.32 cm<sup>2</sup> of the monolayer) while the filters, used for continuous transepithelial resistance measurements, were attached to cylinders (exposing 0.71 cm<sup>2</sup>), and placed in specially modified Ussing chambers. 10 µA was passed across the monolayer via Hg-HgCl electrodes; the voltage change was measured on a Keithley 610C electrometer (Keithley Instruments, Inc., Cleveland, OH); and the electrical resistance was calculated. Measurements taken on filters to be used for chemotaxis experiments were performed rapidly at room temperature in CMEM. The filters were then returned to the incubator. At the time of the experiment, three or four epithelial monolayers were grouped so that the average epithelial resistance of each group was similar. Continuous transepithelial electrical resistance studies were performed on cells bathed in Gey's balanced salt solution (Microbiological Associates, Bethesda, MD) containing 2% (wt/vol) bovine serum albumin (BSA), penicillin (63 U/ml), and streptomycin (138 µg/ml) (Gey's). The studies are done at 37°C in a 95% air/5% CO<sub>2</sub> incubator.

### Preparation of Neutrophils

Citrated venous blood from normal volunteers was separated into a granulocyte-rich fraction by Hypaque/Ficoll (2) and dextran sedimentation techniques (4). Residual erythrocytes were eliminated by hypotonic lysis in 0.2% sodium chloride. This resulted in a cell fraction containing ~98% neutrophils with 98-99% viability as determined from trypan blue exclusion. Before use, neutrophils were suspended ( $5 \times 10^6$  cells/ml) in Gey's.

### Adherence Assay without Antibodies

Neutrophils suspended in Gey's, 1, 10, 25, 50, and 100% serum, or heat-inactivated serum (HIS) were incubated above confluent epithelial monolayers grown in 17-mm diameter Falcon multiwells (Falcon Labware, Oxnard, CA). After a 45-min incubation at 37°C, the unbound cells were removed by washing in Gey's and the bound neutrophils fixed with 2% glutaraldehyde in 0.1 M phosphate buffer. In each multiwell, the number of neutrophils within five 1-mm<sup>2</sup> areas were counted using an inverted microscope at a 125× magnification. Each experimental variable was run in triplicate.

### Adherence Assay with Antibodies

MDCK cells grown to confluency on Falcon Terasaki wells (0.79 mm<sup>2</sup>) were washed in Gey's and incubated 1 h at 37°C in either 10% human serum or

Gey's. The cells were washed in PBS and Gey's and 0.005 ml of the following anti-receptor antibodies were added per well: (a) 50 µg/ml of the Fab fragments of the monoclonal antibody (3G8) which binds and blocks the ligand-binding site of the Fc receptor of human neutrophils (13). The anti-Fc reagent was a gift of Jay Unkless (Rockefeller University, New York, NY). (b) 50 µg/ml of a rabbit polyclonal IgG or a mixture (10 µg/ml each) of Fab fragments of four murine monoclonal antibodies to the C3b receptor (17). Both anti-C3bR reagents were the gift of Dr. V. Nussenzweig (New York University Medical School, New York, NY). (c) 50 µg/ml of the murine monoclonal antibody OKM10 which binds and blocks the ligand-binding site of the C3bi receptor (29). (d) 50 µg/ml of IB4 which binds the C3bi receptor but does not block the binding of C3bi (29) was used as a control. After the addition of the antibodies, 0.005 ml of  $2 \times 10^6$  neutrophils/ml (from the same donor as serum) were added to the wells for 30 min at 37°C. The cells were washed in PBS and fixed 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer. The average number of cells that attached per 0.21 mm<sup>2</sup> of epithelium were determined by counting three random areas per well on a micro-comp data acquisition system (Southern Micro Instruments, Inc., Atlanta, GA). Three wells were counted in each experimental group, and the mean and SEM per group were calculated. Statistical differences between groups were determined by the Student's *t* test.

### Transepithelial Migration

To study transepithelial migration, MDCK monolayers grown on Millipore filters were washed in Gey's and then positioned so that the apical surface faced the neutrophils placed in the upper compartment of a blind well chemotactic chamber. Neutrophils were stimulated to traverse the monolayer during a 45-min incubation period at 37°C, by placing the synthetic chemoattractant, 10<sup>-7</sup> M fMet-Leu-Phe (Peninsula Laboratories, Inc., Belmont, CA) in the lower compartment. In some experiments, fresh human serum (serum) collected from the same donor as the neutrophils, was added directly or after heat inactivation (30 min at 56°C) to both compartments. At the end of an experiment, the filter covered with epithelium and containing the emigrated neutrophils was removed from the chemotactic chamber, fixed, and embedded in Epon 812 for transmission electron microscopy (see below). 1-µm cross-sections of the filter were cut, exposing between 5 and 8 mm of epithelium (diameter, 8 mm). The sections were stained with toluidine blue and examined with the light microscope. The number of neutrophils which bound to the epithelial surface or which had traversed the monolayer and were trapped at the surface of the 0.45-µm pore filter were counted and expressed as the number of neutrophils/mm epithelium. The mean and SEM were calculated for each group of three or four filters and the statistical differences between groups were determined by the Student's *t* test.

### Transmission and Scanning Electron Microscopy

Micropore filters with both neutrophils and epithelial cells were fixed for 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) and washed in 0.1 M phosphate buffer (pH 7.3). For transmission electron microscopy, the filter was postfixated in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3), washed in 0.9% saline, dehydrated, and embedded in Epon 812. Thin sections were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined with the JEOL 100C electron microscope. For scanning electron microscopy the filters were postfixated with osmium using the thiocarbonylhydrazide-osmium tetroxide technique (19), dehydrated, and then critical point dried in a Samdri 790 and examined with the JEOL 100C in the scanning mode.

### Indirect Immunofluorescence

MDCK cells were grown to confluency on 25-mm polystyrene coverslips (Ted Pella, Inc., Tustin, CA). After washing the cells in phosphate-buffered saline (PBS) pH 7.3, the coverslips were cut into eighths and the epithelial cells were incubated 30 min at 37°C in Gey's, serum, or HIS (10, 50, or 100%) or C5a. After incubation, the cells were placed on ice, washed in PBS, and fixed for 15 min in 4% paraformaldehyde/0.05% glutaraldehyde in PBS. The epithelium was again washed in PBS and incubated for 15 min with the following: (a) rabbit antiserum to human C3; (b) goat antiserum to human IgA (alpha chain specific), IgM (mu chain specific), IgG (Fc fragment, gamma chain specific), or C5a; or (c) sheep antiserum to IgE (epsilon chain specific). After washing the epithelium in PBS, the cells exposed to rabbit antiserum were incubated (15 min) with rhodamine-labeled goat antiserum to rabbit IgG (light and heavy chain specific) which had been affinity purified according to the procedure of Herzenberg and Herzenberg (15). The cells incubated with goat or sheep antibodies were incubated (15 min) with either fluorescein-labeled rabbit antiserum to goat or sheep IgG (heavy and light chain specific) that had previously

been absorbed against 4% paraformaldehyde/0.05% glutaraldehyde fixed confluent MDCK cells grown on 35-mm culture dishes (three times for 1 h each). After the second antibody incubation, the epithelium was fixed 15 min in 4% paraformaldehyde/0.05% glutaraldehyde and mounted in 90% glycerol in 10 mM Tris-HCl, pH 8.7. Antibody reagents were obtained from the following sources: C3 (Behring Diagnostics, San Diego, CA); C5a (from Henry Showell) (20); IgA, IgM, IgG, IgE, fluorescein- or rhodamine-conjugated IgG (Cappel Laboratories, Malvern, PA). All coverslips were examined with Zeiss epifluorescence optics and photographed on Tri-X film (Eastman Kodak Co., Rochester, NY).

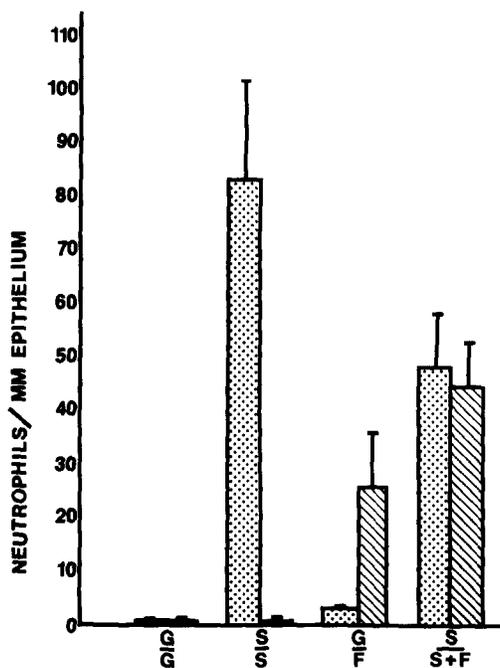
### Preparation of C5a and C5a-enriched Serum

Human C5a was isolated according to the method of Fernandez and Hugli (12). C5a-enriched serum was prepared by incubating 10  $\mu\text{g/ml}$  zymosan A (Sigma Chemical Co., St. Louis, MO) with fresh human serum for 30 min at 37°C. The supernatant was heat inactivated at 56°C for 30 min.

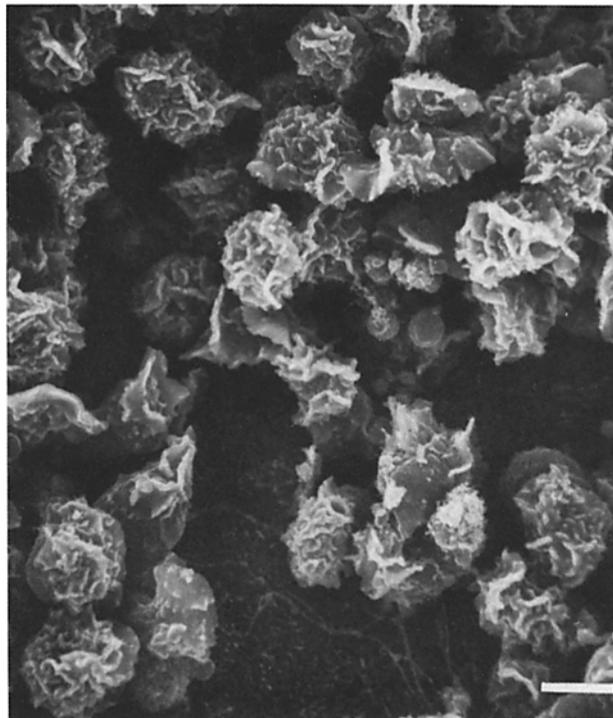
## Results

### Effect of Serum on Transepithelial Migration

The number of neutrophils adhering to and traversing epithelial monolayers in the presence (both compartments) or absence of serum were compared (Fig. 1). Under conditions of random migration (Gey's in both compartments) relatively few neutrophils adhered to or migrated across the epithelium. Under conditions of chemotaxis (Gey's in upper compartment,  $10^{-7}$  M fMet-Leu-Phe in lower compartment), there was a small, but significant increase in the number of neutrophils adhering to the monolayer and a much greater and more significant increase in the number of neutrophils migrating across the epithelium.



**Figure 1.** The effect of serum on neutrophil adherence to and migration across an epithelial monolayer. Human neutrophils were incubated above the kidney epithelium for 45 min in a Blindwell chemotactic chamber with either Gey's (G/G) or 10% serum (S/S) in both compartments or with the addition of  $10^{-7}$  M fMet-Leu-Phe (F) to the lower compartment (G/F, S/S+F). The bars indicate the average number of neutrophils ( $\pm$  SEM) which adhered to (stippled) or migrated across (diagonal lines) the epithelium. The average transepithelial electrical resistance before incubation is  $212.6 \pm 8.9$  ohms/ $\text{cm}^2 \pm$  SEM.



**Figure 2.** Scanning electron micrograph of neutrophils adhering to the apical epithelial surface. Neutrophils in 10% autologous serum were placed in a chemotactic chamber and stimulated to traverse the epithelium for 45 min with 10% serum and  $10^{-7}$  M fMet-Leu-Phe in the lower compartment. The neutrophils adhered to each other and to most, but not all, epithelial cells. Bar, 4.5  $\mu\text{m}$ .

The addition of 10% serum to the buffer in both compartments (random migration) caused over a 400-fold increase in the number of adherent neutrophils but no increase in the number of neutrophils migrating across the monolayer. In contrast, when 10% serum was added to both compartments under conditions of chemotaxis, there was a significant increase in both neutrophil adherence and migration. As can be seen with scanning electron microscopy in Fig. 2, the increase in adherence is due to the binding of neutrophils to the epithelial surface as well as to other neutrophils. Although serum caused a significant increase in neutrophil adherence under conditions of both random migration and chemotaxis, the number of adherent cells was 42% lower under conditions of chemotaxis. This decrease in adherent cells may be attributed to the 43% increase in neutrophils which migrated across the monolayer.

### Target of the Serum Effect

A series of studies were performed to determine if the serum effect was a result of the interaction of serum with neutrophils or epithelium. Neutrophils were preincubated for 40 min in buffer containing 10% serum, washed, and then placed above the epithelial monolayer under conditions of random migration or chemotaxis. In these experiments there was virtually no increase in neutrophil adherence to or migration across the monolayer (data not shown).

In contrast, if the epithelium was preincubated with 10% serum for a similar length of time and then carefully and extensively washed, the increase in neutrophil adherence to

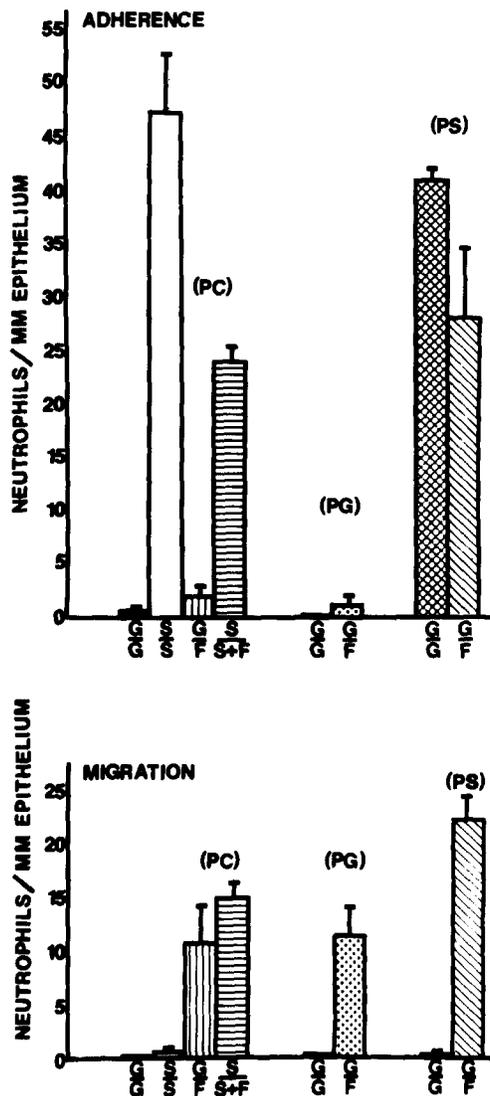


Figure 3. Effect of preincubation of the epithelium on neutrophil adherence and migration. Epithelial monolayers were preincubated in CMEM (PC), Gey's solution (PG), or 10% serum (PS) for 40 min at 37°C and then washed three times in Gey's. Neutrophils were then incubated above the epithelium as described in Fig. 1 with Gey's (G/G) or 10% serum (S/S) in both compartments or with the addition of a chemoattractant,  $10^{-7}$  M fMet-Leu-Phe (F) to the lower compartment (G/F, S/S+F). The bars indicate the average number of neutrophils/mm epithelium ( $\pm$  SEM) which either adhered to or migrated across the epithelium. The average transepithelial electrical resistance before incubation =  $249.7 \pm 11.2$  ohms/cm<sup>2</sup>  $\pm$  SEM.

and migration across the monolayer (under conditions of random migration or chemotaxis) was similar to or higher than that observed when serum was present throughout the experiment (Fig. 3). Transmission electron microscopy of these monolayers revealed a morphologically normal, intact monolayer with neutrophils both adherent to and beneath the epithelium (Fig. 4).

#### Nature of the Serum Effect

(a) *Preabsorption.* The results of the previous study indicated that factors in serum were interacting directly with the epithelium to cause an increase in neutrophil adherence to and

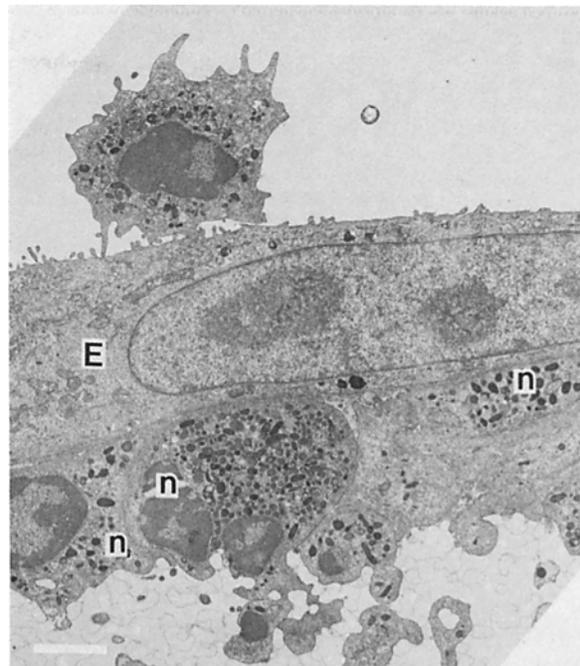


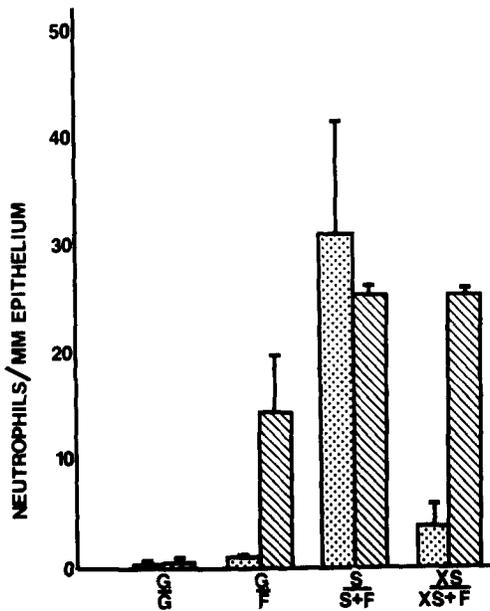
Figure 4. Electron micrograph of a cross section of an epithelial monolayer (E) which had been preincubated in 10% serum for 45 min, washed, and then placed in a chemotactic chamber with neutrophils stimulated to traverse the monolayer by  $10^{-7}$  M fMet-Leu-Phe in the lower compartment. The epithelium remains intact and morphologically normal in appearance. A neutrophil can be seen adhering to the apical surface and neutrophils (n) which have traversed the monolayer are observed beneath the epithelium caught at the surface of the small pore-sized Millipore filter. Bar, 1.6  $\mu$ m.

migration across the epithelium. To determine if these factors could be removed from the serum, 100% serum was incubated at 37°C for 30 min over washed, confluent epithelial cells grown in 60-mm culture dishes (1 ml/dish). Preabsorption of serum resulted in a significant decrease in neutrophil adherence to the epithelial surface without affecting the increase in neutrophil migration across the monolayer (Fig. 5).

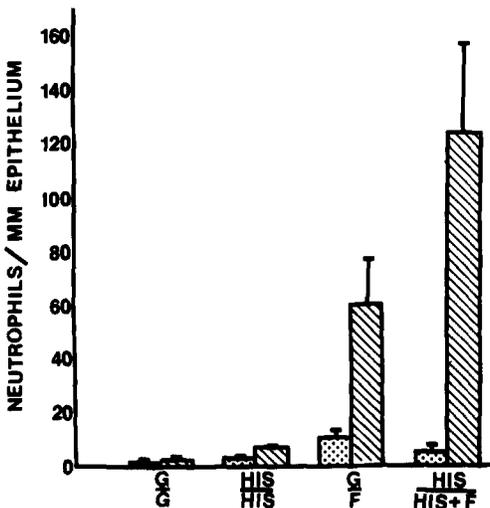
(b) *Heat Inactivation.* To test the possibility that heat-labile components in serum were involved in neutrophil adherence and migration, serum was heat inactivated for 30 min at 56°C. In contrast to 10% serum, 10% HIS only caused a small increase in neutrophil adherence to the monolayer (Fig. 6). However, as observed when serum was present, the presence of HIS also caused a significant increase in the number of neutrophils which traversed the epithelium under conditions of chemotaxis.

(c) *Concentration of Serum and HIS on Neutrophil Adherence.* The neutrophil adherence assay (see Materials and Methods) was used to study the effect of various concentrations of serum and HIS on neutrophil binding to the epithelial surface. Serum caused a marked increase in neutrophil adherence and this increase was significant even with 1% serum (Fig. 7). Neutrophil adherence to the monolayer continued to increase as the percentage of serum increased but began to gradually level off at serum concentrations above 25%.

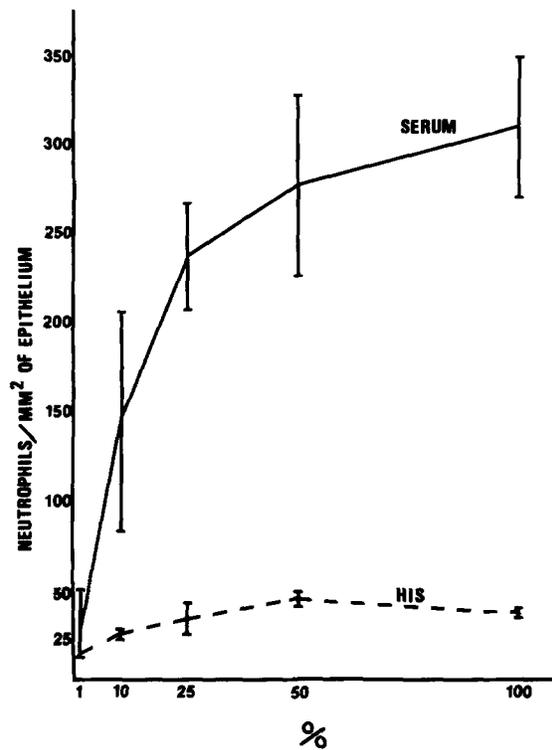
In contrast, serum which had been heated at 56°C for 30 min (HIS) lost a significant amount of neutrophil-binding activity (Fig. 7). However, HIS still caused a significant, although small increase (at 1% and above) in neutrophil



**Figure 5.** Effect of preabsorbed serum on transepithelial migration of neutrophils. Serum (100%) was preabsorbed over epithelial monolayers for 30 min at 37°C. In the presence of 10% preabsorbed serum in both compartments (XS/XS) neutrophils were stimulated to traverse the epithelial monolayer by the presence of the chemoattractant,  $10^{-7}$  M fMet-Leu-Phe (F) in the lower compartment (XS/XS+F). The average number of neutrophils/mm epithelium ( $\pm$  SEM) that adhered to (stippled) or migrated across (diagonal lines) the epithelium was compared to control situations in which Gey's was in both compartments (G/G; random migration) or when Gey's or 10% serum were in both compartments and  $10^{-7}$  M fMet-Leu-Phe was added to the lower compartment (G/F, S/S+F; chemotaxis). The average transepithelial electrical resistance =  $378.6 \pm 20.3$  ohms/cm<sup>2</sup>  $\pm$  SEM.



**Figure 6.** The effect of HIS on the average number of neutrophils/mm epithelium ( $\pm$  SEM) that adhered to (stippled) and migrated across (diagonal lines) the kidney epithelium. The transepithelial migration of neutrophils was examined with either Gey's (G/G) or 10% HIS (HIS/HIS) in both compartments or with the addition of the chemoattractant  $10^{-7}$  M fMet-Leu-Phe (F) to the lower compartment (G/F, HIS/HIS+F). The average transepithelial electrical resistance before incubation =  $103.5 \pm 7.5$  ohms/cm<sup>2</sup>  $\pm$  SEM.



**Figure 7.** Comparison of the number of neutrophils adhering to the epithelial surface in the presence of various concentrations of serum (solid line) and HIS (dotted line). The data is plotted as the mean  $\pm$  SEM of three separate serum experiments and two separate HIS experiments. The average number ( $\pm$  SEM) of neutrophils adhering to 1 mm<sup>2</sup> of epithelial surface under the control conditions (Gey's buffer) of the five separate experiments =  $6.5 \pm 2.0$ .

adherence to the epithelium. This increase never exceeded that observed with 1% serum regardless of HIS concentration.

### Role of C5a

(a) **Chemotaxis.** Studies in other laboratories have suggested complement component C5a may be involved in leukocyte binding to endothelium (6). Consequently, we investigated the role of C5a on neutrophil adherence to and migration across the kidney epithelium. To do this, neutrophils were stimulated to traverse the epithelial monolayers by human C5a ( $10^{-9}$ – $10^{-7}$  M) placed in the lower compartment of the chemotactic chamber (Table I). Both the  $10^{-7}$  M and  $10^{-8}$  M concentration of C5a caused a significant increase in neutrophil transepithelial migration, whereas only the  $10^{-7}$  M concentration caused a small, but significant, increase in neutrophil adherence. The number of neutrophils stimulated to adhere to and to traverse the epithelium by the  $10^{-7}$  M concentration was similar to that caused by 10% zymosan-activated serum which had been heat inactivated for 30 min at 56°C (ZASHIS;  $\sim 3\text{--}4 \times 10^{-8}$  M C5a). Preincubation of the  $10^{-8}$  M concentration of C5a with 40  $\mu$ g/ml of the IgG fraction of goat antiserum to human C5a abolished the chemotactic effect of C5a while 40  $\mu$ g/ml of the IgG fraction of goat preimmune serum did not.

(b) **Chemokinesis.** To mimic the experimental conditions used with serum (serum present on both sides of the epithelium) and to test various concentrations of C5a on neutrophil adherence, neutrophils were incubated above the epithelium

**Table I. Effect of C5a and Anti-C5a on Transepithelial Migration of Neutrophils**

Upper* Lower	Adherence	Migration
G <sup>‡</sup> G	1.0 ± 0.2**	0.1 ± 0.1
G 10 <sup>-7</sup> M C5a	3.6 ± 1.0	80.9 ± 17.2
G 10 <sup>-8</sup> M C5a	0.5 ± 0.3	20.5 ± 3.1
G 10 <sup>-9</sup> M C5a	1.4 ± 0.2	0.9 ± 0.3
G 10 <sup>-8</sup> M C5a + Anti-C5a <sup>§</sup>	1.0 ± 0.3	0.4 ± 0.2
G 10 <sup>-8</sup> M C5a + PIgG <sup>¶</sup>	1.4 ± 1.2	15.6 ± 5.1
G 10% ZASHIS <sup>†</sup>	2.9 ± 0.4	93.9 ± 8.1

\* Solutions in compartments of chemotactic chamber.

<sup>‡</sup> Gey's balanced salt solution with 2% bovine serum albumin.

<sup>§</sup> 40 µg/ml of goat anti-human C5a (IgG fraction).

<sup>¶</sup> 40 µg/ml of goat preimmune IgG fraction.

<sup>†</sup> Zymosan-activated serum, heat-inactivated serum.

\*\* Mean ± SEM.

**Table II. Chemokinesis**

Both compartments*	Adherence	Migration
Gey's	1.0 ± 0.2 <sup>‡</sup>	0.1 ± 0.1
10 <sup>-7</sup> M C5a	0.0 ± 0.0	0.3 ± 0.1
10 <sup>-8</sup> M C5a	0.9 ± 0.5	1.2 ± 0.4
10 <sup>-9</sup> M C5a	1.1 ± 0.5	0.4 ± 0.1
10% ZASHIS <sup>‡</sup>	0.4 ± 0.2	0.4 ± 0.1
10% Serum	90.56 ± 26.7	1.2 ± 0.1

\* Solutions in both compartments of chemotactic chamber.

<sup>‡</sup> Zymosan-activated serum, heat-inactivated serum.

<sup>§</sup> Mean ± SEM.

under conditions of chemokinesis with either 10<sup>-7</sup> M, 10<sup>-8</sup> M, 10<sup>-9</sup> M C5a or 10% ZASHIS in both compartments. Regardless of the concentration of C5a or the presence of 10% ZASHIS, relatively few cells adhered to or traversed the epithelium (Table II). In contrast, as seen previously, when normal 10% serum was present in both compartments there was a significant increase in neutrophil adherence. Thus, while C5a can stimulate neutrophil migration across the epithelium, it has little effect on neutrophil adherence to the monolayer.

#### Localization of Serum Components by Immunofluorescence

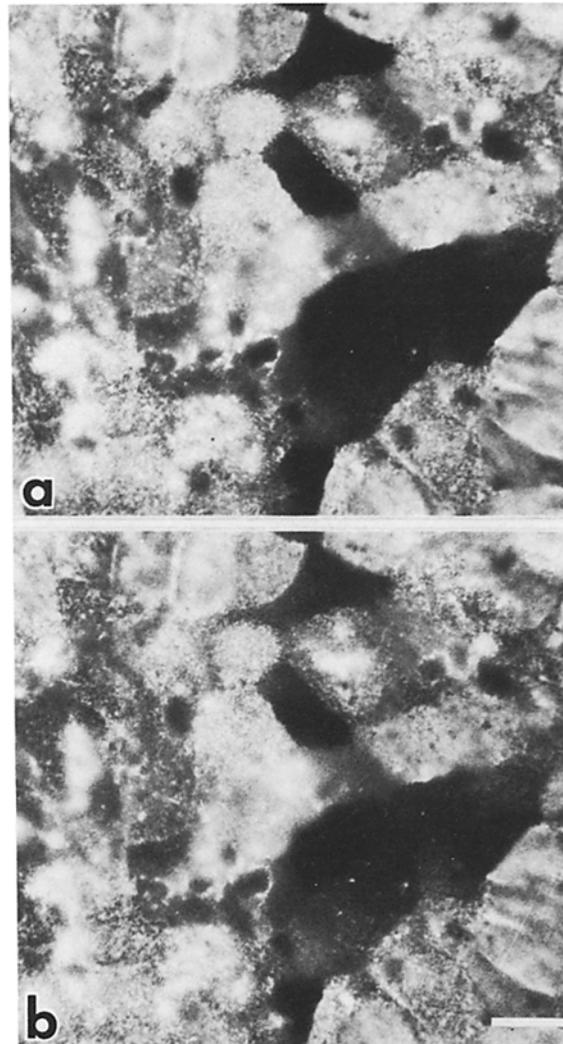
It was clear from some of the previous experiments that components in serum were binding to the epithelium. To identify factors which might be involved, epithelial monolayers were incubated with serum or HIS (10, 50, or 100%), and then tested for the presence of bound C3, IgG, IgA, IgE, and C5a by immunofluorescence. Epithelia incubated with serum and HIS reacted with antibodies to IgG and IgM but not to IgA, IgE, or C5a. C3 could only be detected on epithelia incubated with serum, but not HIS. On all positive coverslips, the majority of cells were immunofluorescent, however, epi-

thelial cells with varying degrees of reactivity were also apparent and ~5% of the epithelial cells did not bind antibodies against IgG, IgM, or C3 (Figs. 8 and 9).

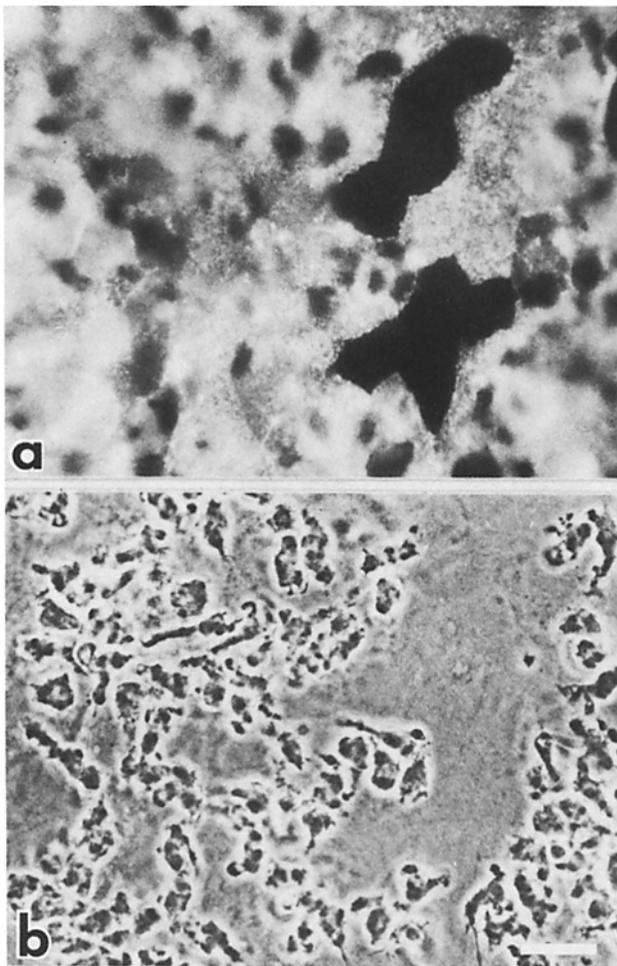
Epithelia incubated with neutrophils suspended in 10% serum or HIS were simultaneously tested for the presence of either IgG or IgM, C3, and bound neutrophils. The majority of epithelial cells incubated in serum-bound IgG or IgM, C3, and neutrophils. The few epithelial cells (5%) which did not bind IgG or IgM also did not bind either C3 or human neutrophils (Fig. 9).

#### Factors Involved in Increased Neutrophil Adherence

It would appear from the above experiments that a heat-labile component in serum which binds to the epithelial surface is



**Figure 8.** Confluent epithelial monolayers were incubated in Gey's containing 10% autologous serum for 45 min at 37°C. After fixation, the distribution of IgG and C3 was detected on the same cells by indirect immunofluorescence microscopy. (a) Goat antiserum to human IgG followed by fluorescein-labeled rabbit antiserum to goat IgG demonstrated that the distribution of cell surface-bound IgG occurred on the majority of the cells. At least five cells in the field ("black holes") did not bind IgG. (b) Rabbit antiserum to human C3 followed by rhodamine-labeled goat antiserum to rabbit IgG indicated C3 on the surface of the epithelial cells with bound IgG. Bar, 16.8 µm.



**Figure 9.** Confluent epithelial monolayers were incubated with human neutrophils ( $1.5 \times 10^6$  cells/ml) in Gey's containing 10% autologous serum for 45 min at 37°C. After fixation the distribution of C3 (a) and neutrophils (b) was examined. Neutrophils did not bind to every epithelial cell but predominately to epithelial surfaces that had bound C3. They did not bind to C3 negative cells. Bar, 16.8  $\mu$ m.

influencing neutrophil binding. To determine if complement component C3 which bound to the epithelial surface was involved in the increased adherence, neutrophils were incubated with epithelial cells in the presence of antibodies to the C3b or C3bi receptors. Rabbit polyclonal antibodies and Fab fragments of monoclonal antibodies against the neutrophil C3b receptor (anti-C3bR) inhibited the binding of neutrophils to epithelial monolayers by 65 and 24%, respectively. Antibodies to the C3bi receptor (OKM10) inhibited binding 55% and the combination of anti-C3b receptor antibodies with anti-C3bi receptor antibody caused a 94% inhibition of binding (Table III). To be sure the anti-complement receptor antibodies did not nonspecifically decrease neutrophil adherence, the effect of these antibodies on neutrophil adherence to plastic was also examined. These antibodies either alone or combined had no effect on neutrophil adherence to a plastic surface (data not shown). The monoclonal antibody to the neutrophil Fc receptor (3G8) or antibody to a region of the C3bi receptor not involved in binding (IB4) had no significant effect on neutrophil binding (data not shown).

### Emigration of Bound Neutrophils

Although in previous experiments neutrophils were observed to adhere to and traverse the epithelium in the presence of serum, it wasn't entirely clear that the emigrating cells came from the population of bound cells. To test this, kidney epithelial cells were preincubated with neutrophils suspended in Gey's or 10% serum for 30 min. The epithelium was washed free of unbound neutrophils. The adherent neutrophils in the presence and absence of 10% serum were then stimulated for 30 min to migrate across the monolayer in response to  $10^{-7}$  M fMet-Leu-Phe. A significant number of the adherent cells separated from the epithelium even in the presence of additional serum, and traversed the monolayer (Table IV).

### Transepithelial Electrical Resistance Studies

In the absence of serum and connective tissue factors, the number of neutrophils which traversed an epithelial monolayer in response to a chemoattractant was dependent on the permeability of the epithelium (7, 22). In this study, a heat-stable factor or factors present in fresh human serum, preabsorbed serum, and HIS were able to affect the epithelium in such a way that significantly more neutrophils migrated across

**Table III.** Antibodies to C3b and C3bi Receptors Prevent Binding of Neutrophils to Serum-treated Epithelial Cells

Preincubation of Epithelium	Added with PMNs	Number of bound cells <sup>1</sup>	Decrease %
Gey's	Gey's	$2 \pm 1^{\dagger}$	99
10% Serum	Gey's	$193 \pm 19$	—
10% Serum	Anti-C3bR*	$68 \pm 8^{**}$	65
10% Serum	Fab Anti-C3bR <sup>‡</sup>	$146 \pm 5^{**}$	24
10% Serum	Anti-C3biR <sup>§</sup>	$86 \pm 15^{##}$	55
10% Serum	Anti-C3bR* + Anti-C3biR <sup>§</sup>	$12 \pm 1^{\dagger}$	94

\* Polyclonal antibody.

<sup>‡</sup> Fab fragments of mixture of four monoclonal antibodies.

<sup>§</sup> OKM10-monoclonal antibody.

<sup>1</sup> Mean number of bound neutrophils/0.2 mm<sup>2</sup>  $\pm$  SEM;  $n = 3$ .

The presence of antibodies significantly reduced the adherence of neutrophils to epithelium preincubated in serum.

<sup>†</sup>  $P < 0.0005$ .

<sup>\*\*</sup>  $P < 0.0025$ .

<sup>\*\*</sup>  $P < 0.05$ .

<sup>##</sup>  $P < 0.01$ .

**Table IV.** Ability of Neutrophils Bound by Immunoglobulins and C3 to Traverse the Epithelium

Preincubate	Upper*		Adherence	Migration
	Lower			
Gey's	G <sup>†</sup> /F <sup>‡</sup>		$0.1 \pm 0.0^{\dagger}$	$0.0 \pm 0.0$
10% Serum	G/G		$24.2 \pm 4.2$	$1.7 \pm 0.0$
10% Serum	G/F		$3.6 \pm 0.7$	$22.7 \pm 2.0$
10% Serum	10% <sup>1</sup> /10%		$32.8 \pm 7.8$	$0.7 \pm 0.2$
10% Serum	10%/10% + F		$11.2 \pm 0.4$	$36.4 \pm 6.5$

\* Solutions in compartments of chemotactic chamber.

<sup>†</sup> Gey's balanced salt solution with 2% bovine serum albumin.

<sup>‡</sup>  $10^{-7}$  M fMet-Leu-Phe.

<sup>1</sup> 10% serum.

<sup>†</sup> Mean  $\pm$  SEM.

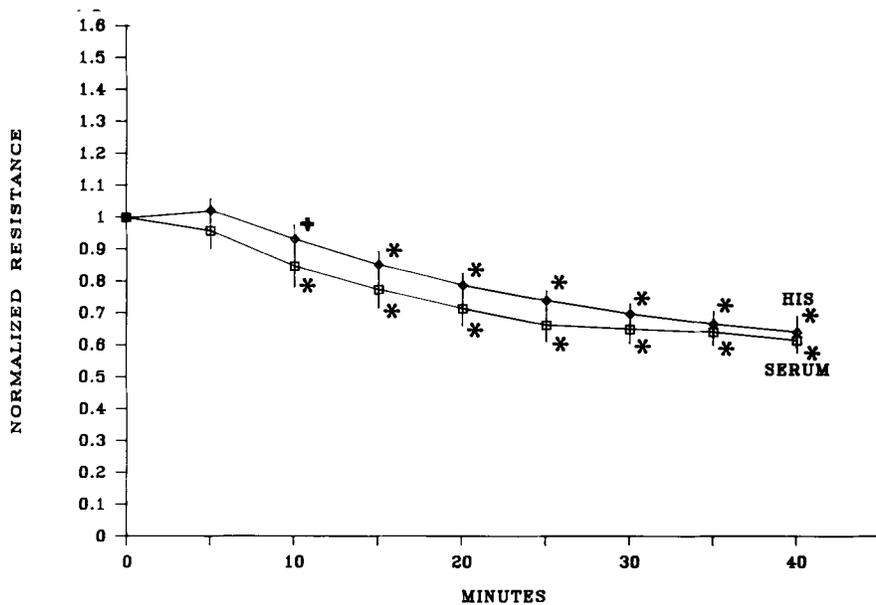


Figure 10. Continuous transepithelial electrical resistance during a 40-min incubation in 10% serum ( $\square$ ) and HIS ( $\diamond$ ). Points shown are from six different epithelial monolayers (two different blood donors for serum, three different blood donors for HIS) normalized to take into account variations in starting resistances. By 10 min there was a significant decrease in epithelial resistance (+ =  $P > 0.005$ ; \* =  $P > 0.0005$ ).

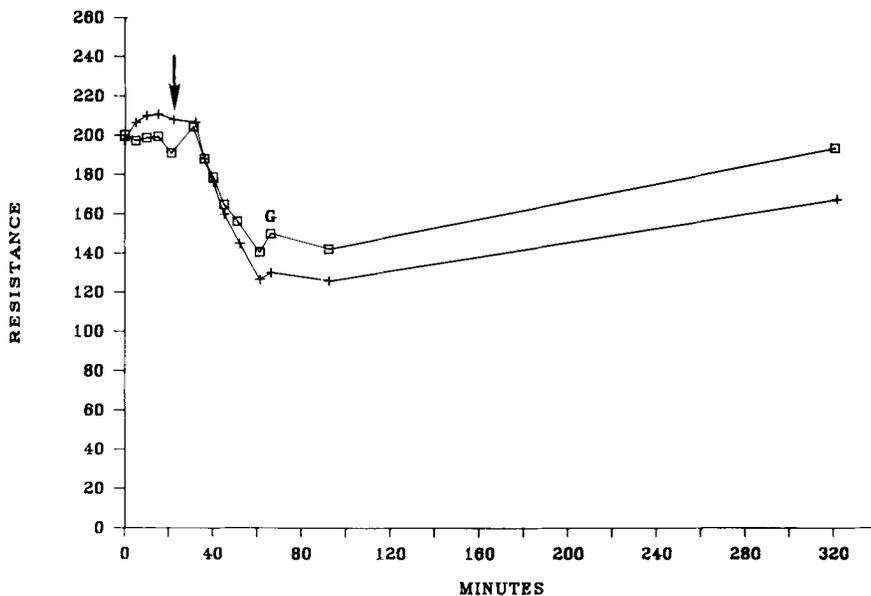


Figure 11. Recovery of transepithelial electrical resistance. 10% serum ( $\square$ ) and HIS (+) were added (arrow) to epithelial monolayers for 45 min and the epithelial electrical resistance fell 26% and 39%, respectively. The monolayers were washed in Gey's (G) and allowed to recover for 5 h. The resistance returned 100% for serum and 81% for HIS.

the monolayer in response to  $10^{-7}$  M fMet-Leu-Phe. It was possible this increase in neutrophil accumulation occurred as a consequence of an increase in epithelial permeability. To determine if a heat-stable factor(s) in serum was affecting the permeability of the epithelium, continuous transepithelial electrical resistance measurements of epithelial monolayers (in the absence of neutrophils) were determined before, during, and after a 40-min incubation ( $37^{\circ}\text{C}$ ) in 10% serum or 10% HIS. In addition, the effect of Gey's and  $10^{-9}$  M to  $10^{-5}$  M fMet-Leu-Phe on the transepithelial electrical resistance was also examined. Neither Gey's nor any concentration of fMet-Leu-Phe (data not shown) effected the permeability of the epithelium. However, a component(s) in both 10% serum and 10% HIS consistently lowered the transepithelial electrical resistance more than 25% (26–52%) in 40 min (Fig. 10). The permeability-increasing factor was unaffected by heating to  $56^{\circ}\text{C}$  since both serum and HIS caused a similar time course of decline in transepithelial electrical resistance (Fig. 10). 5 h after the serum or HIS was removed, the transepithe-

lial electrical resistance of the epithelial monolayer returned to 100 and 81%, respectively, of the starting values (Fig. 11).

## Discussion

A variety of normal and pathological situations result in the emigration of neutrophils out of the blood stream and across a second epithelium. The cascade of events involved in this process and the exact role played by various cells and mediators is difficult to determine in vivo. The present study begins to assess, in an in vitro system, the role of serum and some of its components on the permeability of epithelial occluding junctions as well as on the process of neutrophil adherence to and migration across an epithelial monolayer.

In this system human serum caused an increase in neutrophil adherence to the canine kidney epithelium (Figs. 1 and 2). This increase in binding also occurred if the epithelium had been preincubated with serum (Fig. 3) and it was lost if the serum was preabsorbed over the kidney epithelium before

use (Fig. 5) or heat inactivated (Fig. 6). The immunofluorescence studies indicated that IgG, IgM, and C3 but not IgA, IgE, or C5a bound to the epithelial surface (Figs. 8 and 9). C5a did not appear to be directly involved in the large increase in binding as there was only a modest increase in neutrophil adherence when there was a gradient of the chemoattractant (Table I) or when the attractant was placed on both sides of the epithelium (Table II). However, the presence of IgG and IgM on the epithelial surface caused a small but significant binding of neutrophils to the epithelium (Fig. 7). This increase in binding is more readily apparent in the assay system which did not allow the cells to easily emigrate after they were bound (Fig. 7 vs. Fig. 6). In this situation, neutrophil adherence was probably mediated by the Fc portion of the IgG molecule as neutrophils have a receptor for the Fc domain of IgG (13) and no receptor activity for IgM (18). Neutrophil adherence to the epithelial monolayer coated with immunoglobulins and C3 was significantly greater than neutrophil binding to epithelia with only bound immunoglobulins (Figs. 1, 6, and 7). This is in agreement with previous leukocyte opsonization studies which have demonstrated a synergistic increase in particle binding caused by antibody and complement (9, 18, 21). Through the use of antibodies to the neutrophil C3b, C3bi, and Fc receptor it appeared that neutrophils bound to the epithelial surface predominately by way of their C3 receptors (Table III).

It was of interest that neutrophils bound by both IgG and C3 were able to detach (in the presence and absence of serum) in response to a chemotactic gradient and migrate across the epithelium (Table IV). The interaction of C3 with C3 receptors can be abolished by the action of proteases on C3 (16) but the mechanism by which the neutrophils are stimulated to break a receptor-ligand interaction to respond to another stimulus remains to be determined.

The increase in neutrophil migration across epithelium appeared to be influenced by a heat-stable factor(s) in serum (Fig. 6). This increase in migration did not appear to be due to the increase in neutrophil binding caused by antibody and C3. This was observed in two experiments in which the loss of excessive binding either by using preabsorbed serum (Fig. 5) or by heat inactivation (Fig. 6) did not result in a loss of increased migration. In addition, the increased neutrophil migration does not appear to be caused by complement cytolysis of the epithelial cells as (a) the phenomenon occurred with HIS, (b) electron microscopic examination of monolayers incubated in serum revealed a morphologically normal epithelium, (c) the epithelium appeared viable as assessed by trypan blue dye exclusion (data not shown), and (d) the decrease in transepithelial electrical resistance caused by serum was 100% reversible (Fig. 11). Rather the increase in neutrophil migration appears to be caused by the permeability-increasing properties of the heat-stable factor(s). This factor began to open epithelial-occluding junctions, as assessed by transepithelial electrical resistance measurements, within 10–15 min, and by 40 min had lowered the resistance between 25–52%. This lowering of resistance would increase the number of sites where neutrophils could migrate across the monolayer (7, 22).

In general, the transepithelial electrical resistance of the epithelium lining various organs is considerably higher than the endothelium, a specialized type of epithelium lining blood

vessels. Based on our previous studies (7, 22) it would appear neutrophils would need help to traverse such formidable barriers (1, 5, 10). A substance in serum which could leak out of the blood stream, quickly and temporarily loosen the epithelial occluding junctions through which the neutrophils must pass, would be an important factor in the inflammatory response. The biochemical properties of this substance are currently being investigated and the determination of whether this is a known or unknown permeability factor remains to be clarified.

The authors would like to thank Greg Conyers, Edmund Folkes and Alex Fulop for their expert technical assistance.

This work was supported in part by grants from the National Institutes of Health (AI-16480, AM-30537), Council for Tobacco Research (1661), and New York Heart Association.

Received for publication 14 October 1985, and in revised form 27 January 1986.

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