Degranulating Stimuli Decrease the Negative Surface Charge and Increase the Adhesiveness of Human Neutrophils

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ABSTRACT Chemotactic factors decrease the negative surface charge of neutrophils (polymorphonuclear leukocytes [PMN]) and this has been speculated to be important in PMN margination and aggregation in vivo. PMN adherence and aggregation are also enhanced by degranulation of lysosomal enzymes. To further assess the possible relationship between degranulation, surface charge, adherence, and aggregation, human peripheral blood PMN (isolated by Hypaque-Ficoll and dextran sedimentation) were exposed to the secretagogues ionophore A23187, phorbol myristate acetate, concanavalin A, and chemotactic factors (partially purified C5a or the synthetic peptide f-met-leu-phe) plus cytochalasin B. Surface charge was measured in a cytopherometer. After incubation of PMN with secretagogues, PMN surface charge was decreased to a greater extent than incubation of PMN with chemotactic factors. The decreased surface charge induced by f-met-leu-phe plus cytochalasin B required both extracellular calcium and magnesium. The ionophore A23187-induced surface charge changes were dependent on extracellular calcium but not magnesium whereas the phorbol myristate acetate effect was only partially dependent on Ca^{++} and Mg^{++}. The surface charge changes induced by secretagogues were related to both the amount of lysozyme released and to the increased adhesiveness of cells to plastic surfaces. These observations indicate exocytosis of lysosomal granule contents is associated with decreases in neutrophil surface charge, and there appears to be a correlation between decreases in surface charge and facilitation of neutrophil aggregation and adhesiveness. However, a causal relationship between these events has not been established, and the relationship may be simply temporal.

INTRODUCTION

In 1964, Janoff and Zweifach (1) reported that a cationic protein fraction isolated from rabbit polymorphonuclear leukocyte (PMN)[1] lysozymes caused adhesion and emigration of leukocytes, and it was speculated that alteration of neutrophil surface charge played a role in neutrophil emigration to inflammatory sites. We have reported previously that chemotactic factors cause a small yet significant decrease in human neutrophil negative surface charge (2). This observation has been confirmed (3) and, in several studies, chemoattractants have been shown to cause neutrophil aggregation in vitro and margination in vivo (4–9). Recently it has been reported that degranulating stimuli increase cell aggregation (10, 11) and adhesiveness (12) in vitro. In addition it has been shown that neutrophil adherence to endothelial cells in vitro is enhanced by treatment of neutrophils with neuraminidase and it was suggested their increased adhesiveness may have resulted from electrostatic charge neutralization by removal of sialic acid moieties facilitating cell-cell contact (13). Other studies have implicated decreases in neutrophil surface charge with increased membrane deformability (14), a presumed requirement for migration into tissues (15). These observations prompted the following in vitro studies designed to assess the effect of degranulating stimuli on neutrophil surface charge and adhesiveness.

METHODS

Obtained as follows were: *Escherichia coli* 0127:B8 lipopolysaccharide B (Difco Laboratories, Detroit, Mich.); ^{51}Cr (New England Nuclear, Boston, Mass.); cytochalasin B (cyto B) (Aldrich Chemical Co., Inc., Milwaukee, Wisc.); dimethyl sulfoxide, calcium chloride, magnesium chloride (Fisher

This work was presented in part at the American Association of Immunologists Annual Meeting in Atlanta, Ga., May 1978.

Received for publication 4 June 1979 and in revised form 24 September 1979.

[1] *Abbreviations used in this paper*: Con A, concanavalin A; cyto B, cytochalasin B; EC_{50}, concentration causing half-maximal response; CGD, chronic granulomatous disease; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte.

Scientific Co., Pittsburgh, Pa.); ionophore A23187 (Eli Lilly and Company, Indianapolis, Ind.); phorbol myristate acetate (PMA [Consolidated Midland Corp., Brewster, N. Y.]); concanavalin A (Con A) [two times crystallized in saturated NaCl, Miles-Yeda Laboratories, Rehovot, Israel]); Hypaque- (Winthrop Laboratories, New York) Ficoll and dextran T250 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.); Hanks' balanced salt solution without penicillin, streptomycin, or glutamine and with or without calcium and magnesium (National Institutes of Health (NIH) Media Unit); Gey's balanced salt solution (Microbiological Associates, Walkersville, Md.).

Preparation of human neutrophils. Human leukocytes containing 95–98% neutrophils were separated from heparinized blood drawn from normal volunteers by Hypaque-Ficoll gradient centrifugation (16) followed by dextran sedimentation, osmotic lysis of erythrocytes, and washing in modified Hanks' solution (17). Sterile technique was used throughout the separation procedures. For some studies neutrophils were separated into two populations based on their ability to rosette immunoglobulin (Ig)G-coated erythrocytes as described previously (18). In other studies neutrophils were obtained from three male patients followed at the NIH Clinical Center with well-defined chronic granulomatous disease (CGD) of childhood.

Incubation with degranulating stimuli. Neutrophils were suspended in 2 cm^3 of Hanks' media at a density of 5×10^6/ml. Degranulating stimuli, prepared daily at 100 times the final experimental concentration, were added to the cells in 20-μl aliquots. In experiments with A23187 or PMA, which were dissolved in dimethyl sulfoxide, appropriate controls indicated no effect of dimethyl sulfoxide on the parameters studied. In other experiments the degranulating stimuli included 5–100 μg/ml Con A, or 5 μg/ml cyto B plus either the chemoattractant *E. coli* endotoxin-activated serum, prepared as described previously (19), partially purified C5a (20), or the synthetic peptide chemotactic factor f-met-leu-phe (courtesy of Dr. Elliott Schiffmann, National Institute for Dental Research, NIH). After a 30-min incubation (at 37°C) with the degranulating stimuli, the cells were centrifuged, the supernate saved for enzyme determinations, and the cells were then washed twice in Hanks' media and used for various assays.

Enzyme determinations. Lysozyme activity was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, N. J.) at pH 6.2 according to a turbidometric method (21). Enzyme activity is expressed in terms of micrograms per milligram egg-white lysozyme standard (Worthington Biochemical Corp.). β-Glucuronidase was assayed by measuring the release of phenophthalein from its β-glucuronate (Sigma Chemical Co., St. Louis, Mo.) after a 4-h incubation at pH 4.5 (22). Activity was expressed as micrograms of phenophthalein per 10^7 neutrophils per 4 h. Lactic dehydrogenase was assayed by spectrophotometric measurement of the consumption of diphosphopyridine nucleotide during conversion of pyruvate to lactate (23). Lactic dehydrogenase activity is expressed in Wroblewski units.

Measurement of surface charge. The surface charge of human leukocytes was measured by an electrophoretic mobility technique, previously described (2). For this method, leukocytes were obtained as described above, washed in modified Hanks' solution, and then exposed in suspension to the stimulus for 30 min at 37°C at a concentration of 5×10^6 granulocytes/ml. The cells were then washed twice in Hanks' buffer and twice in 0.13 mM phosphate-buffered (pH 7.2)-5% sorbitol and the electrophoretic mobility then determined with a Zeiss cytopherometer fitted with platinum electrodes (Carl Zeiss, Inc., New York). All measurements were made in the frontal plane at 23°C. For each experimental point 20 determinations were made on 10 different cells, with the second measurement of each cell made after reversal of polarity. Surface charge was calculated as previously described and expressed as micrometers per second per volt per centimeter, and the data throughout the manuscript reflect negative cell surface charge.

Neutrophil adherence and aggregation. The ability of ^{51}Cr-labeled neutrophils to adhere to a plastic surface was quantitated as recently described (12). For these studies, neutrophils were labeled with ^{51}Cr, incubated with degranulating stimuli, and then washed twice with Hanks' buffer. 1 ml of the washed cells (at 3.0×10^6/ml in Gey's media) was then placed into 16-mm diameter wells (Tissue Culture Cluster 24 well, Costar, Div. of Data Packaging Corp., Cambridge, Mass.) followed by a 30-min incubation at 37°C. The wells were then drained and rinsed three times with Hanks' media. The adherent cells were lysed by addition of 1 cm^3 distilled water to the wells followed by a 30-min agitation at room temperature. Adherence was quantitated as the amount of ^{51}Cr released by the adherent cells into the distilled water by counting a 0.5-ml aliquot in a gamma counter. All measurements of adherence were performed in triplicate.

Aggregation was monitored in a standard dual-channel platelet aggregometer/recording system (Payton Aggregation Module, Buffalo, N. Y.), as described previously (4). To a cuvette containing a siliconized stirring bar revolving at 900 rpm was added 1 ml of neutrophils at a density of 10^7/ml in Gey's balanced salt solution. The recorder was calibrated so that full scale was 5 mV and maximal light transmission occurred with cells at a density of 8×10^6/ml. After a 2-min delay, to allow equilibration of the cells, 10 μl of stimulus was added.

Purification of neutrophil granules. For some studies neutrophils were incubated with neutrophil granule lysates. Neutrophil granules were separated by continuous sucrose gradients using methods adapted from previous reports (24, 25). Neutrophils suspended in 0.34 M sucrose were lysed in the presence of sodium heparin (The Upjohn Company, Kalamazoo, Mich., 1,000 U/10^7 neutrophils/ml), by repeatedly aspirating and expressing cell suspensions through a 20-gauge spinal needle. The extent of lysis was followed by phase microscopy and cell counts in a hemocytometer chamber until >85% of cells were lysed. Lysates were then passed serially through two 13-mm diameter polycarbonate filters (Neuroprobe, Nuclepore Corp., Pleasanton, Calif.), the first having a 5-μm pore size and the second a 2-μm pore size. 5-ml granule-rich lysates were layered over continuous sucrose gradients in $1 \times 3\frac{1}{2}$-in. cellulose nitrate tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Sucrose gradients were prepared with a continuous gradient maker (MSE, Inc., Westlake, Ohio) using heavy and light sucrose solutions of specific gravities 1.28 and 1.12, respectively. After centrifugation, gradients were pumped out from the bottom through a 0.8-mm × 14-cm rigid tube put directly through the gradient and held in a fixed position against the bottom of the centrifuge tubes. Gradients were pumped out at a constant rate (1 ml/min; LKB peristaltic pump-1200, LKB Instruments, Inc., Rockville, Md.) and fractionated into 1.2-ml aliquots. Turbidity of the fractions was monitored by measuring optical density at 450 nm. Aliquots of these fractions were then exposed to a final concentration of 0.1% Triton X-100 (Rohm and Hass Co., Philadelphia, Pa.) and enzyme activities in the fractions were assayed. Aliquots of granule-rich fractions were also diluted in 0.34 M sucrose and recentrifuged at 50,000 *g* for 45 min at 4°C. The granule suspensions were then sonicated and their enzyme content was assessed as described above; their protein content was determined by the Lowry method (26). With this procedure three granule preparations are obtained and have been

labeled A, B, and C as previously described (24). Based on enzyme content and morphology, granule preparations A and B have been considered a heterogenous population of azurophil or primary granules and granule preparation C corresponds to the specific or secondary granules.

Determination of superoxide production. Superoxide production was measured by monitoring the superoxide dismutase inhibitable reduction of ferricytochrome *c* to ferrocytochrome *c*. PMN suspensions (5×10^6/ml in Hanks' balanced salt solution) were incubated at ambient temperature in 0.1 mM ferricytochrome *c*, type VI (from horse heart [Sigma Chemical Co.]), with or without the indicated stimuli in quartz cuvettes. For some experiments superoxide was generated by incubating xanthine (0.1 mM, Sigma Chemical Co.) plus xanthine oxidase (0.05 U/ml, Sigma Chemical Co.) for 15 min with PMN. Control experiments contained superoxide dismutase (100 μg/ml, Miles Laboratories, Inc., Elkhart, Ind.). The amount of superoxide generated was determined by repeatedly scanning at 1-min intervals the absorption spectrum of the samples from 540 to 560 nm (model 552, Perkin-Elmer Corp., Instrument Div., Norwalk, Conn) and the point and amplitude of maximum absorption (≅550 nm) was recorded. Nanomoles of cytochrome *c* reduced were calculated from the maximal increase in absorbance measured using an absorption coefficient of 21.1 mM per cm (reduced-oxidized) (27). Occasionally the total cytochrome *c* concentration was checked by reduction of the samples with dithionite. Superoxide production was calculated as the difference between the amount of cytochrome *c* reduction obtained between parallel samples in the absence and presence of superoxide dismutase. The results are expressed as nanomoles of superoxide dismutase-inhibitable cytochrome *c* reduced per 5×10^6 neutrophils.

Cell viability. Cell viability after exposure to the various compounds was monitored by their ability to exclude trypan blue dye (Grand Island Biological Co., Grand Island, N. Y.) and measurements of the cytoplasmic enzyme lactic dehydrogenase released into the extracellular environment. Normally >95% are estimated to be viable with these assays and under the conditions studied, none of the stimuli used affected cell viability.

Statistical analyses. Standard error was used as an estimate of variation and the means of different experiments were compared with the unpaired, two-tailed Student's *t* test. For some experiments straight lines were fitted to the data by the method of least squares (28).

RESULTS

Effect of degranulating stimuli on neutrophil surface charge. As shown in Fig. 1, a small yet significant reduction in neutrophil surface charge was seen when neutrophils were exposed to the chemoattractant f-met-leu-phe ($P < 0.05$ at 1 nM vs. 0 f-met-leu-phe). The EC_{50} (concentration causing half-maximal response) was 0.3 nM. When cyto B was added to the neutrophils before the f-met-leu-phe, an enhancement of the maximum surface charge reduction was seen and the EC_{50} for f-met-leu-phe changed to 50 nM (Fig. 1).The respective EC_{50} for surface charge reduction by f-met-leu-phe in the absence or presence of cyto B are in close agreement with the previously published chemotactic and degranulating activities of f-met-leu-phe under these conditions (12, 29). It is also apparent from Fig. 1 that incubation of human neutrophils with cyto B without

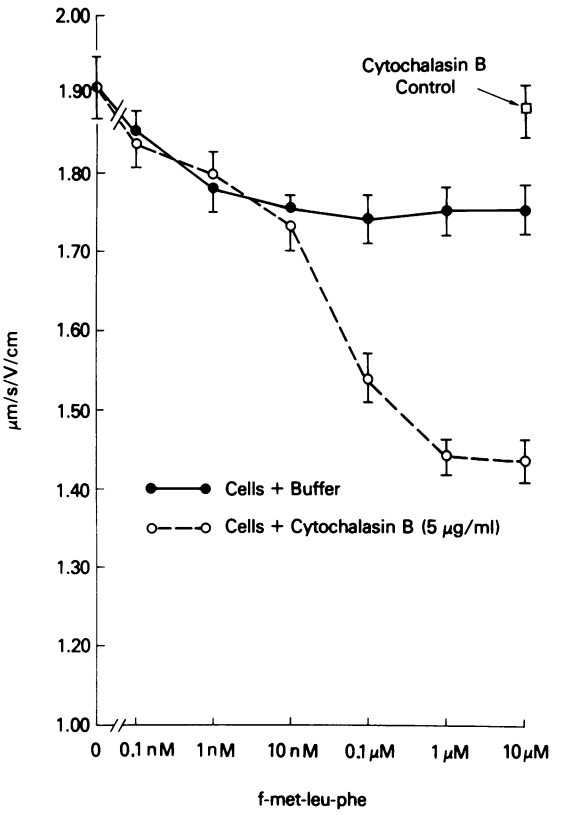

FIGURE 1 Effect of secretion on neutrophil surface charge. Human neutrophils were incubated for 30 min in the indicated concentrations of f-met-leu-phe with or without 5 μg/ml cyto B. After the incubation the cells were washed twice in Hanks' media and three times in phosphate-sorbitol buffer before surface charge measurements (see Methods). Data are mean ±SEM negative surface charge of 20 measurements in 10 cells, one experiment.

f-met-leu-phe had no significant effect on surface charge. Similar data to that shown with f-met-leu-phe were obtained when neutrophils were incubated with *E. coli* endotoxin-activated serum or partially purified C5a with or without cyto B (data not shown).

The effect of incubating neutrophils with three other degranulating stimuli (ionophore A23187, PMA, and Con A) are shown in Fig. 2. Each stimulus produced a significant reduction in surface charge in concentrations which have been shown previously (12) to induce lysozyme secretion. In addition, as shown in Fig. 3, the magnitude in reduction in surface charge by the various stimuli was linearly related to the amount of lysozyme released from the neutrophils in response to the various secretagogues used ($P < 0.01$, linear regression analysis). No correlation was seen between the amount of β-glucuronidase released and the decrease in surface charge.

The decrease in surface charge could not be related to depletion of a population of aggregating cells with

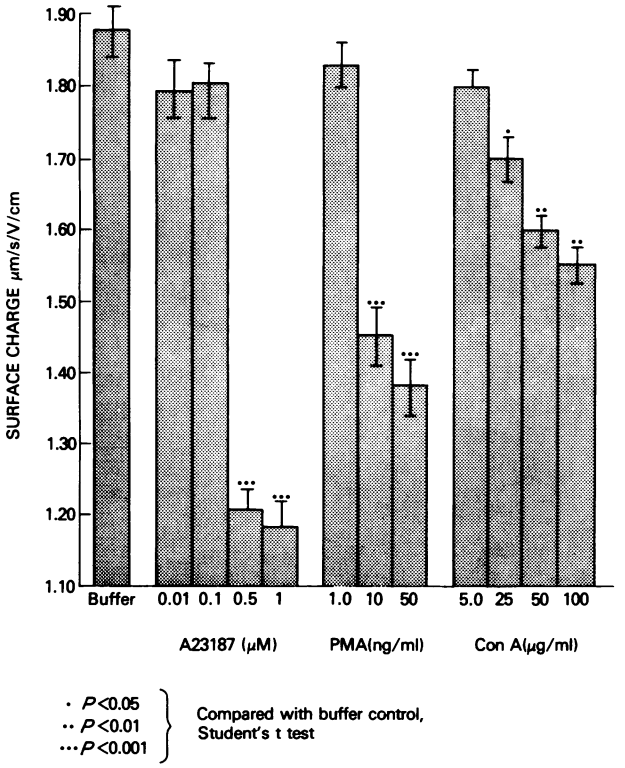

FIGURE 2 Effect of degranulating stimuli on neutrophil surface charge. Cells were incubated in the indicated concentrations of ionophore A23187, PMA or Con A. After incubation the cells were washed twice in Hanks' media and three times in phosphate-sorbitol buffer before surface charge measurements (see Methods). Data are the mean±SEM negative surface charge of three separate experiments.

high electronegativity from the assay system because the neutrophil surface charge after degranulation was less than the charge of any cells before exposure to secretagogue. In addition, when cells were exposed to conditions of minimal secretion, with small changes in surface charge (i.e., 1.92±0.02 μm/s/V/cm for control vs. 1.80±0.03 μm/s/V/cm for cells stimulated with 5 ng/ml PMA), the negative surface charge of small cell aggregates (two to four cells) was also reduced (to 1.74 ±0.02 μm/s/V/cm, $P < 0.01$), indicating that aggregating cells do have surface charge changes. Related studies were performed with neutrophils separated into subpopulations on the basis of their ability to rosette IgG-coated erythrocytes. The surface charge of the population of IgG-rosetting cells, which are particularly susceptible to stimuli of aggregation,[2] did not differ from the surface charge of cells which do not rosette with the IgG-coated erythrocytes and aggregate poorly (1.82 ±0.03 vs. 1.79±0.04 μm/s/V/cm, respectively, three experiments). Furthermore, the IgG-rosetting cells showed a large reduction in surface charge when incubated with secretagogues (not shown).

The time-course for secretion of lysozyme and decrease in surface charge was also studied. For these studies the time required for cell washing after incubation with secretagogues precluded precise time points for the surface charge changes. Nonetheless, it is apparent that the time-course for the decrease in surface charge by A23187 was similar to the release of lysozyme (Fig. 4). Time-course studies were also done with PMA and f-met-leu-phe (1 μM) plus cyto B (5 μg/ml) and the decrease in surface charge paralleled the release of lysozyme.

Calcium and magnesium requirements for surface charge changes. The requirement of calcium and magnesium for the decreases in surface charge were studied. As shown in Fig. 5 with f-met-leu-phe plus cyto B as the stimulus maximal reduction in surface charge was seen when 1.5 mM Ca^{++} and 1.0 mM Mg^{++} were present. These concentrations of Ca^{++} and Mg^{++} were selected after preliminary dose-response studies indicated they produced a maximal response. The surface charge changes in the presence of Ca^{++} and Mg^{++} were greater than with either ion alone, even when the total divalent cation concentration was maintained at 2.5 mM ($P < 0.05$ for comparison of f-met-leu-phe plus cyto B with 1.5 mM Ca^{++} and 1.0 mM Mg^{++} vs. either 2.5 mM Mg^{++} or 2.5 mM Ca^{++}). The calcium and

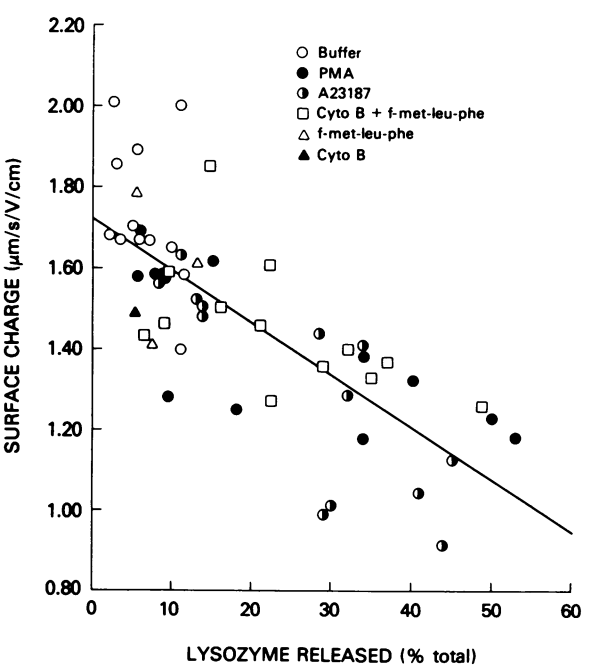

FIGURE 3 Relationship between neutrophil surface charge and the amount of lysozyme secreted by the stimuli used in three separate experiments. Each data point represents the mean negative surface charge obtained from 20 measurements made in 10 cells (see Methods).

[2] Klempner, M. S., and J. I. Gallin. Unpublished observations.

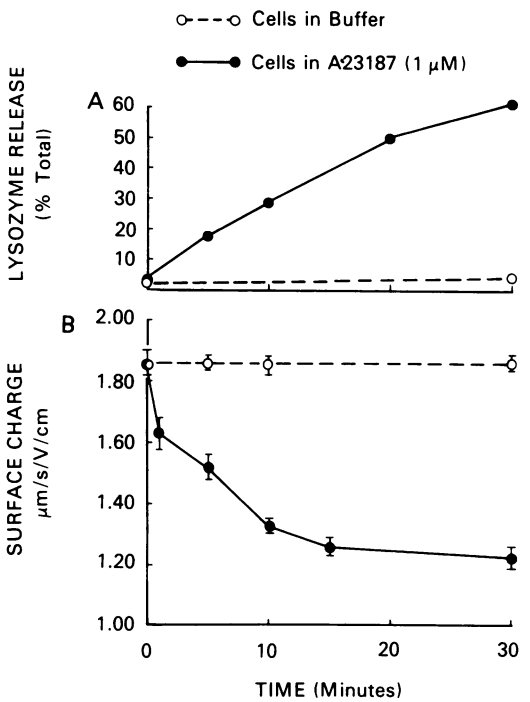

FIGURE 4 Time-course of the effect of A23187 on lysozyme release (A) and surface charge changes (B). 2-ml cells (5×10^6/ml) were incubated in triplicate tubes with A23187 for the indicated times, centrifuged and then washed in Hanks' media. Lysozyme measurements are the amount of enzyme released into the media (mean of three replicates). The negative surface charge data represent 20 measurements on 10 cells for each of the three separate replicate cell preparations.

magnesium requirements for surface charge changes are similar to those required for PMN chemotaxis through cellulose nitrate filters (20, 30) or for degranulation (31).

In contrast to the calcium and magnesium requirements for the maximum decrease in surface charge by f-met-leu-phe plus cyto B the surface charge reduction seen with ionophore A23187 was dependent on extracellular calcium but not magnesium (Fig. 6). The differences in the surface charge of neutrophils incubated without A23187 or with A23187 but without Ca^{++} and Mg^{++} or A23187 without Ca^{++} but with Mg^{++} were not significant ($P > 0.05$ for each comparison). Increasing extracellular Mg^{++} to 5 mM did not change these results. A significant reduction in surface charge occurred with cells incubated with A23187 in the presence of Ca^{++} but without Mg^{++} ($P < 0.001$); this was not significantly different from the decrease in surface charge seen with cells incubated with A23187 with Ca^{++} and Mg^{++} ($P > 0.05$). Other studies in which the calcium concentration was varied indicated that the maximal decrease in surface charge by A23187 (0.01–1 μM) was seen at 1.0 mM Ca^{++} with no further decreases seen through 5 mM extracellular Ca^{++} (data not shown). The Ca^{++} and Mg^{++} requirements for PMA reduction in surface charge indicated that although small yet significant decreases in surface charge occurred in the absence of extracellular calcium or magnesium, maximal reduction in surface charge was achieved in the presence of both calcium and magnesium, similar to that seen with f-met-leu-phe and cyto B (Fig. 5).

Effect of incubating neutrophils with secreted products or lysates of isolated neutrophil granules. Incubation of fresh neutrophils with postsecretory supernates (obtained from cells incubated for 30 min with 0.1 μM A23187 or 10 ng/ml PMA) caused a small yet significant ($P < 0.05$) reduction in surface charge.

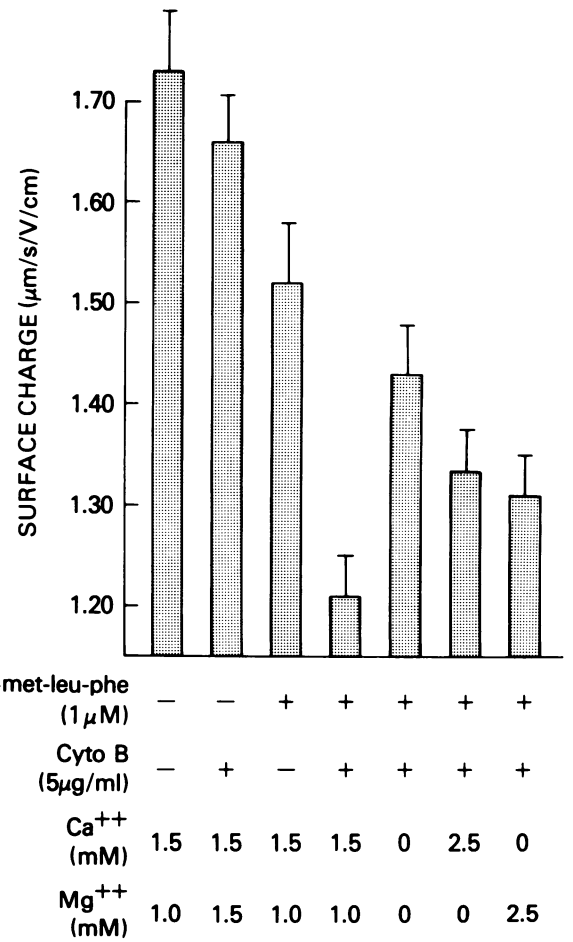

FIGURE 5 Calcium and magnesium requirements for decreases in neutrophil negative surface charge by f-met-leu-phe plus cyto B. 3-ml cells (3×10^6/ml) were incubated in Hanks' media lacking Ca^{++} and Mg^{++} to which the indicated amount of Ca^{++} or Mg^{++} was added. The final reaction system, containing the indicated amount of f-met-leu-phe or cyto B, was incubated 30 min at 37°C. The cells were then washed twice in Hanks' media, three times in phosphate-sorbitol buffer, and then the surface charge measured. Data are mean±SEM negative surface charge of four separate experiments.

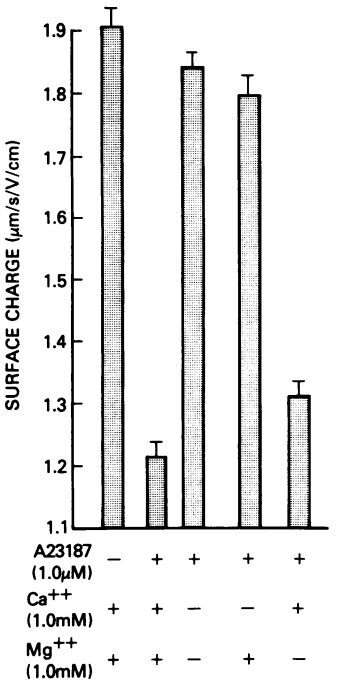

FIGURE 6 Calcium and magnesium requirements for decreases in neutrophil negative surface charge by ionophore A23187. 3 ml of cells (3×10^6/ml) was incubated in Hanks' media lacking Ca^{++} and Mg^{++} to which the indicated amount of Ca^{++} and Mg^{++} was added. The final reaction system, containing the indicated amount of A23187, was incubated 30 min at 37°C. The cells were then washed twice in Hanks' media, three times in phosphate-sorbitol buffer and then the surface charge measured. Data are the mean±SEM of four separate experiments.

Postsecretory supernates obtained when higher concentrations of stimuli were used (1 μM A23187; 50 ng/ml PMA) caused a larger decrease in surface charge. From these preliminary experiments it was not possible to conclude whether the decrease in surface charge after incubation with postsecretory supernates was an effect of secreted products on the fresh cells or residual stimuli present in the media.

To assess this question further, neutrophils were incubated with sonicates of neutrophil granules isolated by sucrose gradient centrifugation (Methods). As shown in Table I granule sonicates containing the specific granules "C", whose contents are most accessible for extracellular release (24, 32), caused insignificant decreases in surface charge under the conditions shown. In contrast incubation of neutrophils with granule lysates of azurophil granule preparations A and B caused a small but significant decrease in surface charge (Table I). Because no decreases in neutrophil surface charge were seen with the "C" granules under these conditions, other experiments in which the concentration of lysozyme in the C granule preparation was increased 10-fold over that detected in postsecretory supernates were performed. A significant decrease in neutrophil surface charge was observed with this concentrated C granule preparation (from 2.04±0.03 to 1.74±0.03 μm/s/V/cm, $P < 0.05$). Thus, these data indicate that although neutrophil granule contents can decrease neutrophil surface charge, the large decrease in surface charge seen after selective degranulation of specific granules (i.e., by 0.5 μM A23187 or 20 ng/ml PMA) would appear to involve other mechanisms unless the products of these granules remained tightly adherent to the plasma membrane during secretion resulting in a very high concentration of secreted product at the cell surface.

Effect of superoxide generation on surface charge changes. Each of the stimuli used to decrease the negative surface charge stimulates superoxide generation. To assess the possible effect of superoxide on neutrophil surface charge two sets of experiments were performed. The first experiments were done with neutrophils from three subjects with CGD whose cells did not produce significant superoxide. The surface charge of the CGD PMN was decreased appropriately with 0.1 μM f-met-leu-phe plus 5 μg/ml cyto B (from 1.93 ±0.09 to 1.32±0.09 μm/s/V/cm for three different CGD preparations vs. from 1.92±0.09 to 1.35±0.06 μm/s/V/cm for three studies with normal cells). In a second series of experiments normal neutrophils were incubated with a superoxide generating system (xanthine plus xanthine oxidase). Under these conditions significantly more superoxide dismutase-inhibitable cytochrome *c* reducing activity was generated (100 nM cytochrome *c* reduced/15 min) compared with the nanomolars of cytochrome *c* reduced when PMN are incubated with 1 μM A23187 (3.0), 50 ng/ml PMA (1.8), 5 μg/ml cyto B plus

TABLE I
Effect of Neutrophil Granule Lysates on Surface Charge

Granule fraction*	Surface charge‡	*P*
	μm/s/V/cm	
None	2.04±0.03	—
Azurophil (primary) granules		
A	1.80±0.02	<0.05
B	1.75±0.02	<0.001
Specific (secondary) granules		
C	1.91±0.03	>0.05

* The lysozyme content of the granule preparations in microgram egg-white lysozyme equivalents per milliliter was A = 14.2, B = 10.3, and C = 16.2 and the β-glucuronidase content in micrograms phenolphalein released/4 h per ml was A = 11.9, B = 10.2, and C = 3.4.

‡ Surface charge after incubation of neutrophils with granule lysates from one neutrophil preparation for 30 min at 37°C. (see Methods) Mean±SEM of 20 determinations on 10 cells, one experiment.

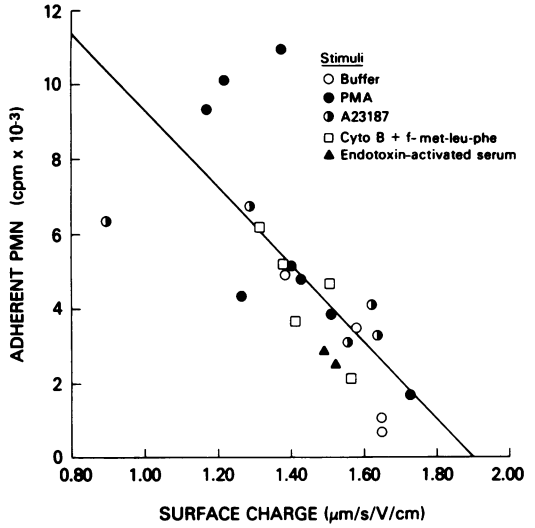

FIGURE 7 Relation between PMN surface charge and the adhesiveness of ^{51}Cr-labeled cells to a plastic surface. 8 cm^3 of ^{51}Cr-labeled cells (3 × 10^6/ml) were incubated for 30 min at 37°C with buffer or with various concentrations of the stimuli shown by the symbols. The cells were then washed, divided, and used for adherence and surface charge studies. Each experimental point represents the mean of three adherence measurements where the total counts per minute of cells added to adherence wells was 3,584±328. Negative surface charge measurements are the mean of 20 determinations on 10 cells.

0.1 μM f-met-leu-phe (1.8), or buffer (0.5) ($P < 0.01$ for each comparison). No superoxide was generated with xanthine or xanthine oxidase controls. A small but significant ($P < 0.05$) reduction in surface charge was seen with xanthine plus xanthine oxidase (from 1.79±0.07 to 1.66±0.09 μm/s/V/cm). However, this decrease in surface charge was seen with xanthine oxidase controls and was significantly ($P < 0.01$) less than the reduction in surface charge induced by degranulating stimuli.

Relationship between neutrophil surface charge and adhesiveness. Degranulating stimuli have been reported to increase cell adhesiveness (12) and promote neutrophil aggregation (10, 11). It has been suggested that decreases in surface charge may facilitate these events (7). As shown in Fig. 7 when cells were incubated with the various stimuli shown, a linear relation ($P < 0.01$, linear regression analysis) existed between the cell surface charge and their adhesiveness to plastic surfaces; the lower the surface charge the greater the adhesiveness.

In related studies, neutrophil adhesiveness to each other (aggregation) was monitored in a platelet aggregometer. In these studies we confirmed earlier reports (10, 11) that degranulating stimuli facilitate neutrophil aggregation. Cyto B enhanced PMN aggregation in response to a preparation of C5a; similar data were obtained with 1 μM f-met-leu-phe plus 5 μg/ml cyto B. Cyto B alone had no effect on aggregation and the aggregation response was not altered if the cyto B were added just before or just after the chemoattractant. 50 ng/ml PMA, 1 μM A23187, and 50 μg/ml Con A also caused PMN aggregation (not shown).

DISCUSSION

These experiments show that when human peripheral blood neutrophils are incubated with degranulating stimuli there is a reduction in the negative surface charge of the cells as monitored with an electrophoretic mobility technique. In addition, the magnitude of the decrease in surface charge is greater than that produced by incubating neutrophils with chemotactic factors in the absence of cyto B. A linear relationship exists between the decrease in surface charge and the amount of lysozyme released. Furthermore, the calcium and magnesium requirements for the decrease in surface charge are similar to the reported requirements for secretion (31, 33).

The data support the concept that decreases in neutrophil negative surface charge and degranulation are related events. However, with the present data it is not possible to conclude whether the surface charge changes are related to the initiation or are a consequence of degranulation of whether they are merely coincidental. It is possible the submembranous cation deposits we have reported recently in human neutrophils exposed to chemotactic factors (34) are related to the observed decrease in surface charge. Alternatively, local deposition of positively charged proteins such as lysozyme on the cell membrane could produce a decrease in surface charge. Studies of the effect of sonicated granules isolated by sucrose density centrifugation of neutrophil homogenates on surface charge (Table I) are compatible with both possibilities.

It is tempting to relate the surface charge changes to neutrophil function. Lichtman has shown that in the bone marrow immature neutrophils have a high density of negative surface charge that decreases during cell maturation (14). The decrease in surface charge during cell maturation was related to increased membrane deformability (14) which is probably required for normal chemotaxis (15). The recent demonstration that neutrophil degranulation accompanies chemotaxis in vitro and exudation in vivo (35) would be compatible with the concept that decreases in surface charge associated with degranulation is integrally related to alteration of cell deformability during diapedesis.

In addition to possibly being important in membrane deformability, decreases in neutrophil negative surface charge may facilitate neutrophil margination and adherence to endothelial surfaces. This concept was apparently first suggested by Abramson in 1927 based

on studies with horse leukocytes (36) and again in 1964 by Janoff and Zweifach (1) working with rabbit cells. More recently, Fehr and Jacob postulated surface charge neutralization might be important in neutrophil aggregation and subsequent margination in vivo (7). This concept has been strengthened by the report that sialidase, which decreases negative surface charge (2), facilitates neutrophil adherence to endothelial cells (13). The linear relationship between surface charge changes induced by secretagogues and cell adhesiveness would support these conclusions.

However, it needs to be emphasized that although these data suggest that reduction in negative surface charge is required for cell-cell contact and diapedesis in vivo, proof is still lacking. Indeed, although there appears to be a correlation between degranulation, surface charge changes, and aggregation it is possible the relationship is simply temporal and not causally related. Each of the degranulating stimuli studied initiates other intracellular functions including assembly of microtubules and microfilaments both of which may be important in PMN adhesion to the endothelium. Thus, additional studies monitoring neutrophil surface charge and degranulation during margination in vivo and assessing the role of surface charge of neutrophils and endothelial cells during cell-cell interaction in vitro are required to determine what role, if any, electrostatic forces play in neutrophil margination and diapedesis.

ACKNOWLEDGMENTS

The author thanks Dr. Bruce Seligmann for performing the superoxide assays, Dr. Laurence Corash for help using the platelet aggregometer, Dr. David Alling for valuable statistical advice, and Mrs. Rhoda Hubert for excellent technical assistance.

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