Electrophoretic Mobility and N-Acetyl Neuraminic Acid Content of Human Normal and Leukemic Lymphocytes and Granulocytes

By MARSHALL A. LICHTMAN AND ROBERT I. WEED

The surface charge of mammalian cells is considered to be one of several factors determining the balance between attractive and repulsive forces that operate between the cell periphery and other surfaces of potential contact.1,2 The surface-charge density of the leukocyte may be important in determining surface characteristics such as adhesiveness, deformability, and pseudopod formation. Variations in the latter characteristics are likely to be of major importance in determining the ability of leukocytes to remain in or leave the bone marrow, to marginate and exit from the vascular compartment, and to ingest particulate material.

Human leukocytes, like other mammalian cells examined, carry a net negative surface charge at physiologic pH.3 Furthermore, it has been shown that the negative charge of leukocytes can be reduced by the action of the enzyme, neuraminidase (n-acetyl neuraminidate glycohydrolase)4; hence, a portion of the surface charge is considered to be due to ionized carboxyl group of n-acetyl neuraminic acid (NANA) in the electrokinetic zone of the leukocyte periphery.

The studies to be described were performed to compare the electrophoretic mobility of leukemic and normal mature granulocytes, immature granulocytes and lymphocytes in order to determine whether electrophoretic mobility is correlated with cell age or with the leukemic state; and, to determine the contribution of neuraminidase susceptible groups to the electrophoretic mobility of differing leukocyte types. Furthermore, the NANA content of granulocytes and lymphocytes and the fraction of leukocyte NANA susceptible to neuraminidase treatment were examined.

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Prepared from Vibrio cholera obtained from Sigma Chemical Co.
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METHODS

Normal polymorphonuclear granulocytes (PMNG), normal and leukemic immature granulocytes, and small lymphocytes and leukemic myeloblasts were isolated as previously described. Normal and leukemic immature granulocytes from blood and bone marrow of patients with CGL, and from bone marrow of subjects without hematologic disease were obtained by filtration of the samples through glass wool at 35°C prior to removal of erythrocytes by lysis with 150 mM NH₄Cl. Immature granulocytes were thereby separated from PMNG which adhered to the glass wool. The resultant immature granulocytes were washed with Hanks balanced salt solution (HBSS), and were composed of myeloblasts, promyelocytes, and myelocytes when examined by phase microscopy or by Wright's stain.

Cell electrophoretic mobility was measured at 23°C in a rectangular cuvette as described by Fuhrmann and Ruhenstroth-Bauer. The time in seconds (sec.) required for leukocytes to traverse 147 microns (µ) in a gradient of 17.5 volts per centimeter (v./cm.) at a constant current of 30 µA was recorded and expressed as µ/sec./v./cm. Twenty to 50 cells were examined. Each cell was measured twice with reversal of polarity after the first measurement. In each experiment, measurements were made at the anterior and posterior stationary level of the electrophoresis cuvette.

Isolated leukocytes were incubated in HBSS with and without ouabain, 5 X 10⁻⁴ M, or with and without neuraminidase* (50 units/mL) for as long as two hours. Monovalent cation content of leukocytes was measured as previously described. The pH of leukocyte incubations was measured with a radiometer pH meter and maintained at 7.30-7.50. In the case of leukocyte suspensions containing neuraminidase, incubations were maintained at pH 7.10-7.30. After incubation, leukocytes were washed once in a solution of Sorensen's phosphate buffer and sorbitol (1 part 1/15 M Sorensen's phosphate buffer and 4 parts 5% Sorbitol), pH 7.20, prior to electrophoresis in the same medium. Conductivity of the phosphate buffer used for electrophoresis studies was measured at the time of each experiment.

N-acetyl neuraminic acid was measured by the method of Aminoff. Erythrocyte ghosts prepared by osmotic lysis and leukocyte suspensions were exposed to acid hydrolysis in 0.1 N H₂SO₄ at 80°C for 60 min. The hydrolysate was centrifuged at 50,000 × g., and NANA measured in the supernatant. Leukocyte suspensions treated with neuraminidase were centrifuged at 500 × g., and the supernatant assayed for NANA. The residual NANA of neuraminidase-treated leukocytes was measured by exposing leukocytes to acid hydrolysis after enzyme treatment. Studies indicated that acid hydrolysis of sonicated or intact leukocytes released similar amounts of NANA. Erythrocyte suspensions treated with neuraminidase were centrifuged at 500 × g., and the supernatant assayed for NANA. Isoamyl alcohol extraction of acid hydrolysates of erythrocyte ghosts, leukocytes, and NANA standards was performed with each experiment, and correction for the 5–10 per cent extraction of NANA by isoamyl alcohol was thereby made. Correction was made for the interference of desoxyribose as suggested by Warren. Studies indicated that neuraminidase did not interfere in the assay of NANA. Trichloracetic acid precipitation of solutions to be measured for NANA resulted in low optical-density readings unless the hydrogen ion concentration of the solution was reduced with sodium hydroxide.

RESULTS

Leukocytes suspended in phosphate–sorbitol buffer were examined by phase contrast microscopy and were resuspended in autologous serum and Wright's stained smears examined. Leukocytes appeared intact by both methods of observation.

Normal and leukemic leukocytes have potassium-sodium ratios of approximately one at the time of their isolation. Incubation in Hanks Balanced Salt Solution resulted in potassium-sodium ratios of 3 to 4, whereas incubation in HBSS containing 5 × 10⁻⁴ M ouabain for two hours produced further loss of
potassium and gain in sodium with potassium-sodium ratios of approximately one half after two hours. Leukocytes after incubation in HBSS and after incubation in the presence of ouabain had mobilities which did not differ from freshly isolated leukocytes; hence, variations in intracellular monovalent cation produced by isolation techniques did not influence electrophoretic mobility.

Human erythrocytes had an electrophoretic mobility of $2.13 \pm 0.03$ (1 S.E.) $\mu$/sec./v./cm. under the conditions of measurement described. The electrophoretic mobility of the small lymphocyte was significantly less than that of erythrocytes, and significantly greater than that of PMNG's, and these differences are similar to previous observations by others. However, the mobility of small lymphocytes from patients with CLL did not differ significantly from that of normal small lymphocytes (Table 1).

| Table 1.—Electrophoretic Mobility of Human Blood Cells |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                               | Normal                        | CLL                           |
| Erythrocytes                  | PMNG                          | Lymphocytes                   | Lymphocytes                   |
| $(n = 16)$                    | $(n = 9)$                     | $(n = 9)$                     | $(n = 10)$                    |
| $2.13 \pm 0.017$             | $1.38 \pm 0.041$             | $1.81 \pm 0.046$             | $1.76 \pm 0.033$             |

Values represent mean $\pm$ S.E. of electrophoretic mobility of 30-50 leukocytes from each of $n$ donors expressed in $\mu$/sec./v./cm.

| Table 2.—Electrophoretic Mobility of Human Blood Cells Treated with Neuraminidase |
|-------------------------------|-------------------------------|--------------------------|--------------------------|
|                               | Normal                        | CLL                      | Leukemic                 |
| Erythrocytes                  | PMNG                          | Lymphocytes              | Lymphocytes              | Myeloblasts              |
| $(n = 9)$                     | $(n = 3)$                     | $(n = 3)$                | $(n = 3)$                | $(n = 3)$                |
| $0.53$                        | $0.58$                        | $0.62$                   | $0.64$                   | $0.59$                   |
| $(0.34-0.67)$                 | $(0.54-0.60)$                 | $(0.52-0.64)$            | $(0.56-0.68)$            | $(0.56-0.62)$            |

Mean and range: $\mu$/sec./v./cm.

After PMNG and normal and leukemic small lymphocytes were treated with neuraminidase, a 70 per cent reduction in lymphocyte, and a 55 per cent reduction in PMNG, mobility occurred; and both cell types had similar mobilities (Table 2). Ninety-five per cent of the reduction in electrophoretic mobility of leukocytes produced by neuraminidase was achieved after 30 min. incubation at pH 7.2. No change in mobility occurred when leukocytes were incubated for similar periods in the absence of neuraminidase. Erythrocytes treated with neuraminidase had a 75 per cent reduction in electrophoretic mobility and had similar mobilities to those of leukocytes after enzyme treatment.

A decrease in adhesiveness to glass surfaces was characteristic of normal and leukemic immature granulocytes and myeloblasts, and allowed their separation from PMNG after passage through glass wool. Immature granulocytes from patients with CGL and from normal bone marrow had similar mobilities (Table 3). Although in five of eight cases of acute granulocytic leukemia the population of myeloblasts studied had mobilities similar to that of immature granulocytes from normal bone marrow, in three cases the mobilities were more rapid. PMNGs from patients with CGL had slower mobilities than immature
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Table 3.—Electrophoretic Mobility of Immature Granulocytes

<table>
<thead>
<tr>
<th></th>
<th>Leukemic Myeloblasts</th>
<th>CGL</th>
<th>Normal Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.01</td>
<td></td>
<td>1.86</td>
<td>1.84</td>
</tr>
<tr>
<td>(1.74–2.34)</td>
<td></td>
<td>(1.74–2.00)</td>
<td>(1.74–1.96)</td>
</tr>
</tbody>
</table>

Median and range: μ/sec./v./cm.

Table 4.—Electrophoretic Mobility of Immature and Mature Granulocytes

<table>
<thead>
<tr>
<th></th>
<th>Normal Blood (n = 9)</th>
<th>Bone Marrow (n = 4)</th>
<th>CGL * Blood (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature Granulocytes</td>
<td>1.84</td>
<td>(1.74–1.96)</td>
<td>1.78</td>
</tr>
<tr>
<td>PMNG</td>
<td>1.38</td>
<td>(1.16–1.60)</td>
<td>1.38</td>
</tr>
</tbody>
</table>

* Immature granulocytes and PMNG from same subjects.

Median and range: μ/sec./v./cm.

Granulocytes from the same subjects, and the mobility of PMNG from patients with CGL was similar to that of normal PMNG (Table 4). In one subject with CGL, the electrophoretic mobility of PMNGs prior to therapy (1.41 μ/sec./v./cm.) was very similar to the mobility of his PMNG during a remission induced with chemotherapy (1.44 μ/sec./v./cm.). Myeloblasts treated with neuraminidase had a 70 per cent reduction in mobility and had mobility similar to other neuraminidase-treated leukocytes (Table 2).

PMNG NANA content was 2½ times that of lymphocytes per milliliter of cells (Table 5). Approximately 60 per cent of total cellular NANA was released from both PMNGs and lymphocytes after treatment with neuraminidase (Table 6). Since the number of lymphocytes per milliliter of packed cells is 1.8 times that of PMNGs, the NANA content of the lymphocyte is about one-fifth that of the PMNG (Table 7). Hence the increased density of NANA at the electrokinetic layer of the lymphocyte as compared to that of the PMNG is not made apparent by measurement of total cellular NANA content which is higher in the PMNG than in the lymphocyte.

DISCUSSION

The electrophoretic mobility of single cells is considered to be a measure of the cell's surface-charge density. Since a number of assumptions must be made

Table 5.—N-Acetyl Neuraminic Acid Content of Human Leukocytes

<table>
<thead>
<tr>
<th></th>
<th>Normal PMNG (n = 5)</th>
<th>Lymphocytes (n = 1)</th>
<th>CLL Lymphocytes (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.13</td>
<td>0.44</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>(0.82–1.60)</td>
<td></td>
<td>(0.41–0.54)</td>
</tr>
</tbody>
</table>

Median and range: μM./ml. cells.
Table 6.—N-Acetyl Neuraminic Acid Content of Human Leukocytes: Effect of Neuraminidase

<table>
<thead>
<tr>
<th></th>
<th>Untreated Cells</th>
<th>Neuraminidase-treated Cells</th>
<th>Supernatant of Neuraminidase-treated Cells</th>
<th>Per cent NANA Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMNG</td>
<td>1.60</td>
<td>0.75</td>
<td>0.86</td>
<td>54</td>
</tr>
<tr>
<td>CLL Lymphocytes</td>
<td>0.52</td>
<td>-</td>
<td>0.33</td>
<td>63</td>
</tr>
<tr>
<td>CLL Lymphocytes</td>
<td>0.51</td>
<td>0.35</td>
<td>0.31</td>
<td>63</td>
</tr>
</tbody>
</table>

μM./ml. cells.

Table 7.—N-Acetyl Neuraminic Acid Content of Human Blood Cells (Mean Values)

<table>
<thead>
<tr>
<th></th>
<th>Erythrocyte</th>
<th>Lymphocyte</th>
<th>PMNG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cellular NANA (μM./cell)</td>
<td>5.0 x 10^-11*</td>
<td>14 x 10^-11</td>
<td>56 x 10^-11</td>
</tr>
<tr>
<td>NANA (neuraminidase-susceptible)</td>
<td>4.9 x 10^-11</td>
<td>8.4 x 10^-11</td>
<td>39 x 10^-11</td>
</tr>
<tr>
<td>Approximate Surface Area (μ²)</td>
<td>160 †</td>
<td>200 †</td>
<td>300 †</td>
</tr>
<tr>
<td>Approximate Surface Area (μ²)</td>
<td>160 †</td>
<td>340 §</td>
<td>1090 §</td>
</tr>
</tbody>
</table>

* Amount released from hydrolysis of erythrocyte ghosts.
† Surface area of human red cell as measured by Ponder.64
§ Approx. leukocyte surface area = surface area of erythrocyte per leukocyte

...to arrive at a numerical value for charge density, investigators have cautioned that broad confidence intervals must be placed around such estimates.10,11 Nevertheless, the presence of significantly different rates of mobility when two cell types are compared under identical conditions of measurement indicates a difference in the surface charge density of the compared cells.11

Human leukocytes migrate anodally in an electric field and hence have a net negative surface charge. The nature of the electrokinetic behavior of the leukocyte at pH 6.5-12,13 combined with the reduction in mobility by purified neuraminidase and demonstration of release of NANA after neuraminidase treatment provides evidence that a large portion of the ionogenic groups contributing to the surface charge of leukocytes is due to NANA. The residual charged groups have not been defined, although recent studies have suggested that ionized phosphate groups of ribonucleic acid located at the surface of certain cells may be responsible for a portion of the residual charge.14-16 If this proves to be the case in the human lymphocyte, it will raise provocative questions about the potential role of surface ribonucleic acid in the transfer of information between specific human cells. Haydon and Seaman have suggested that an α-carboxylic acid of a protein-bound amino acid may account for the residual charge of neuraminidase-treated erythrocytes.17

The surface charge, or the molecules that contribute to the surface charge, may affect migration of cells into inflammatory foci,18 deformability of cell periphery, and thereby pseudopod formation, motility and phagocytosis.19,20
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circulatory patterns of cells,21-23 cell–cell aggregation and disaggregation,24,25
the antigenic nature of the cell surface,26,27 and the movement of ions across
cell membranes.28 The possible role of molecular groups contributing to the
cell surface charge in the behavior of malignant cells has also been discussed.26,27,29,30

Coman observed that epithelial cancer cells would disaggregate more read-
ily than their normal counterparts.31 Since that time, investigators have com-
pared surface characteristics of neoplastic and normal cells since possible
differences might provide insight into differences in their behavior. Ambrose
and co-workers presented evidence that animal tumor cells have a higher sur-
face-charge density, as estimated from their electrophoretic mobility, than
their normal counterparts.32-34 Defendi and Gasic showed by histochemical
techniques that acid polysaccharides susceptible to neuraminidase were in-
creased at the surface of polyoma-transformed hamster cells.35 However, Cook
and Jacobson found that the surface-charge density of mouse leukemic lympho-
blasts is lower than that of normal mouse lymph cells, although these in-
vestigators suggested qualitative differences in the ionogenic groups contribut-
ing to the surface charge of the leukemic leukocytes.36 Simon-Reuss and co-
workers, after studying various cell types, concluded that no generalizations
could be made regarding the relationship of electrokinetic properties of cells
and malignancy.37 Vassar, after examining a broad variety of human cells,
found that tumor cells did not exhibit an increased surface-charge density
as compared to homologous cells.38,39 Kimuri and co-workers could find no
difference in the hexosamine and sialic acid content of malignant as compared
to normal cells.40

Ruhrenstroth-Bauer and co-workers have suggested that human leukemic
leukocytes have more rapid electrophoretic mobility than normal leukocytes.4,41,42
Our findings do not confirm this; rather, they are in agreement with
the observations of Robineaux and Bazin,3 who found that normal and leukemic
granulocytes and lymphocytes have similar electrophoretic mobility. Recently,
Meharishi and Thompson have reported that human normal and leukemic
small lymphocytes do not differ in electrophoretic mobility.33 Consequently,
it appears inappropriate to conclude at this time that increased surface-charge
density, as reflected by electrophoretic mobility measurements, is a constant
or even frequent concomitant of neoplastic transformation in human cells. In
animal studies, as well, there are exceptions to the direct relationship between
cell surface charge and malignancy.36 More important, in instances where
the parallel has been close, a causal relationship has not been established.
To further support such a relationship, the effect of reduction in surface charge
by neuraminidase on the malignant potential of cells would be of importance,
although this is difficult to study because of the capacity of cells to restore sur-
face sialic acid. However, observations have indicated that there is no effect on
the growth potential or malignant behavior of neuraminidase-treated cells.43,44

Ben-Or and co-workers observed an increased surface-charge density in
regenerating rat liver cells as compared to adult cells, and suggested that the
increased surface-charge density of tumor cells may be a manifestation of
growth potential or growth rate.45 Subsequently, Heard and co-workers
observed a higher surface-charge density in embryonic as compared to adult mouse fibroblasts; similarly, the presence of a high surface-charge density in immature granulocytes as compared to mature granulocytes may be a reflection of the membrane structure of cells with growth potential.

Mayhew and O'Grady, and Brent and Forrester, have shown that parasynchronized human cells in suspension culture have an increased surface-charge density at the time of mitosis. Further investigation has shown that the increased electrophoretic mobility of premitotic cells is due to charged groups susceptible to neuraminidase and possibly to ribonuclease. Kraemer has suggested, based on critical assumptions, that the increased surface charge density of dividing cells is due to conformational or terminal complex changes of NANA containing macromolecules, rather than new synthesis. Although this transient increase in surface-charge density is of interest and potentially important in facilitating the separation of daughter cells, it does not explain the increased surface charge of immature granulocytes or lymphocytes in vitro since these cells are not entering cell division.

The decrease in negative surface-charge density of immature granulocytes after maturation to PMNGs may play a role in the release of granulocytes from the marrow, and may be central to the surface interactions of the PMNG. Gottschalk has suggested that the density of negative charges contributed by peripherally oriented NANA carboxyl groups may determine the flexibility of the underlying protein core of a salivary mucoprotein, since the proximity of negative charges may, by electrostatic repulsion, tend to restrict the deformability of the underlying protein core. Weiss has drawn an analogy to the situation in mammalian cells by suggesting that the tendency to a reduced surface-charge density in phagocytic and motile cells like monocytes and PMNGs, contributes to their ability to form low-radius-of-curvature probes which may be the fundamental initiating displacement of the cell surface prior to cell movement or to the engulfment stage of phagocytosis. Indeed, Weiss has demonstrated an increase in the contact and engulfment stages of phagocytosis by monocytes treated with neuraminidase. Increased deformability of neuraminidase-treated monocytes was also suggested by micropipette experiments. Weiss has further suggested that the presence of an NANA lyase, found by Fishkin and co-workers, to increase in activity in turpentine-induced rat granuloma, might play a role in inflammatory areas by enhancing phagocytosis. The identification of neuraminidase in mammalian cell lysosomes is of particular interest in this regard.

The result of studies of total NANA content of leukocytes is similar to that in Ehrlich ascites tumor cells, in that cellular NANA content does not correlate with electrophoretic mobility. This finding indicates that in the leukocyte, as in other cells, the density of NANA molecules contributing ionized carboxyl groups to the electrokinetic zone of the cell is not correlated with total cellular NANA although, within a given cell type, there appears to be a correlation between cellular NANA content and changes in cell volume during mitosis. The larger amount of NANA in the PMNG as compared to the lymphocyte may reflect the increased external membranous area of the PMNG, as well as an increased internal membranous surface area due to the more
Numerous organelles of the PMNG. It should be emphasized that surface area estimations based on the comparison of NANA released by neuraminidase-treated leukocytes as compared to erythrocytes (Table 7), are made on the assumption (1) that NANA released by neuraminidase reflects external surface NANA and (2) that surface NANA is similar in density per unit surface area in the cell types compared. Therefore, the estimate of surface area by this method is a rough approximation. However, it indicates that the surface area of the lymphocyte and PMNG based on the assumption of a smooth sphere is a minimal estimate (Table 7). This is also indicated by recent microscopic pictures of the leukocyte surface. Comparative studies of leukocyte surface-related characteristics must consider the effect that the difficulty in expressing data per unit surface area may produce.

The proportion of leukocyte NANA released after neuraminidase treatment is similar to that of other nucleated cells and, as in other cell types, the NANA released by neuraminidase does not necessarily correlate with the reduction in surface charge as measured by cell electrophoresis. A number of reasons have been proposed to explain this observation. It is likely that some sialic acid groups that are susceptible to neuraminidase do not contribute to the electrokinetic zone of the cell. Such a possibility is heightened by the observation of Nordling and Mayhew which indicates that neuraminidase may enter nucleated cells and release NANA on surfaces other than the outer cell membrane. In the erythrocyte where factors that could explain the discrepancy are minimized, a better, although imperfect, relationship does exist between NANA release by neuraminidase and reduction in electrophoretic mobility. It is clear from our observations and those cited above, that surface-charge density due to NANA cannot be predicted from studies of cellular NANA content, but depend on measurement of electrophoretic mobility of cells.

Despite the theoretical arguments favoring an important role for the surface-charge density of the cell in the interaction of the cell and its environment, studies of such relationships have been conflicting and difficult to interpret, in part because of the alterations produced by in vitro observations, the inability to study the interaction of all relevant variables, and the inability to measure other than average surface characteristics. Although critical experiments are still needed to establish the causal role of surface-charge density or the molecules contributing to the surface-charge density in various areas of cell-cell and cell–extracellular interactions, it is likely that the differences in surface-charge density of leukocytes of different type and morphologic age reflect a fundamental change in the structure of the cell periphery which is causally related to differences in cell peripheral functions.

**Summary**

Studies have been conducted to determine the electrophoretic mobility and the N-Acetyl Neuraminic Acid (NANA) content of different human leukocyte types. Leukemic granulocytes and lymphocytes do not differ significantly from their normal counterparts in rate of electrophoretic mobility. An absolute comparison between leukemic and normal myeloblasts could not
be made; however, populations of leukemic myeloblasts were often similar in mobility to normal immature granulocytes. Immature granulocytes had a significantly higher surface-charge density than PMNGs, and this is due primarily to differences in NANA carboxyl groups contributing to the electrokine

tic surface of the cell. The small lymphocyte has a surface-charge density more similar to that of immature granulocytes and myeloblasts than to that of PMNGs. The surface-dependent behavior of different leukocyte types may be related, in part, to the density or arrangement of NANA molecules at the cell periphery; alternatively, the distribution of surface NANA may be a reflection of other differences in molecular arrangement of the cell membrane which are important in determining functional capacities. Total cellular NANA is greater in the PMNG than the lymphocyte, and this is likely to be a reflection of the larger surface area of external and internal membranes of the PMNG. These data also suggest that the surface area of the PMNG and lymphocyte is not reflected by the assumption of a smooth sphere. Neither total NANA nor neuraminidase-susceptible NANA can be used as an estimate of comparative NANA-dependent surface-charge density which must depend on electrophoretic mobility measurements with and without neuraminidase treatment.

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