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The Electrophoretic Mobility of Normal and Leukaemic Cells of Mice

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1. The pH–mobility relationships for saline-washed cells from a mouse strain of acute lymphoblastic leukaemia were examined before and after treatment with lower aldehydes, diazomethane and neuraminidase (EC 3.2.1.18). 2. The content of sialic acid released into the supernatant fluid of neuraminidase-treated cells was measured. 3. The stability of the charge-determining structures to temporary changes in environment (pH and ionic strength) was established. 4. Similar measurements were made on lymph-node cells obtained from non-leukaemic mice (a resistant and a leukaemia-susceptible strain were examined). 5. It is deduced that both the malignant and the non-malignant cell possess two dissociable acid functions at the cell surface, a carboxyl group of sialic acid and another acidic group(s), probably carboxyl, of pK 3·0–4·5. The malignant cells, however, have a basic dissociable function not present in the non-malignant types. 6. Suggestions are made as to how the difference in surface chemistry may be related to the problem of malignancy.

It has been suggested (Ambrose, James & Lowick, 1956) that the electrokinetic properties of the cell surface may change when a cell undergoes malignant transformation. In a mouse sarcoma the negative electrophoretic mobility of the cells has been shown (Purdom, Ambrose & Klein, 1958) to increase as they acquired more malignant properties. Many studies have been made in an attempt to elucidate the nature of cell-surface ionogenic groups. The view that ionizable phosphate groups, probably associated with a complex phospholipid system, are mainly responsible for the electrokinetic properties of cells (Winkler & Bungenberg de Jong, 1941) must now be revised, since more recent studies (Cook, Heard & Seaman, 1961; Eylar, Madoff, Brody & Oncley, 1962) have demonstrated that sialic acid anions are responsible for a major portion of the surface negative charges on erythrocytes and various tumour cells (Cook, Heard & Seaman, 1962; Forrester, Ambrose & Macpherson, 1962).

The object of the present investigation was to characterize and compare the chemical nature of the surface charges of normal lymph cells and their malignant counterparts. The cells were examined in suspensions prepared from normal lymph nodes of a strain of healthy AK mice and from the leukaemic lymphoblasts of mice of the same AK strain with an acute lymphoblastic leukaemia. The behaviour of the normal and leukaemic cells was studied by electrophoresis coupled with suitable chemical and enzymic treatment. The lymph cells of another, related, strain of mice that were resistant to leukaemia were also investigated for comparison.

MATERIALS AND METHODS

Solutions. The Tyrode solution, containing 0·1% glucose, used for preparing the cell suspensions was made by the method of Paul (1960). Physiological saline was a solution of 0·145 M-NaCl made 0·3 mM with respect to $NaHCO_3$, pH 7·2 ± 0·2 (Heard & Seaman, 1960). Saline–sorbitol media of low ionic strength and iso-osmotic aqueous solutions of HCl and NaOH for use in adjusting the pH of a given suspension of cells were prepared as described by Heard & Seaman (1960).

Purified neuraminidase (EC 3.2.1.18, lot no. 1165B, 966B) from *Vibrio comma* (formerly *Vibrio cholerae*) filtrate was obtained from Behringwerke A.-G. (Marburg/Lahn, Germany) as an aqueous solution containing 500 units/ml., where 1 unit of activity is defined as the amount of enzyme that releases 1 μg. of N-acetylneuraminic acid in 15 min. at 37° from α_1-acid glycoprotein in an appropriate medium at pH 5·5. This preparation is stated by the manufacturer to be free of lecithinase C and neither proteases nor aldolase activity could be demonstrated; we showed that quantities of enzyme in excess of those used in this study were free of proteolytic enzyme activity against Azocoll (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) for 1 hr. at 37° and pH 7·3.

A 2% (w/v) solution of acetaldehyde and a 1·5% (w/v) solution of formaldehyde in physiological saline (Heard & Seaman, 1961) were prepared as described by Simon-Reuss, Cook, Seaman & Heard (1964).

Diazomethane was generated from a suspension of N-methyl-N-nitrosotoluene-p-sulphonamide (Diazald; Ralph N. Emanuel Ltd., London, S.E. 1) in diethyl ether, by

dropwise addition to ethanolic KOH, as described by De Boer & Backer (1954). The ethereal solution of diazomethane was standardized by the addition of a known excess of benzoic acid and back-titration with NaOH.

Paraformaldehyde, paraldehyde, sodium arsenite, sorbitol and 2-thiobarbituric acid were of laboratory reagent grade (British Drug Houses Ltd., Poole, Dorset.); Erythrosin B was adsorption-indicator grade (Hopkin and Williams Ltd., Chadwell Heath, Essex); all other reagents, unless otherwise stated, were of A.R. grade. For the preparation of all solutions, distilled water was obtained from an electrically heated automatic still (Manesty Machines Ltd., Liverpool) and was redistilled immediately in a Pyrex glass still fitted with a suitable spray trap; this water was used within 12 hr. of distillation. All solutions were in equilibrium with the atmosphere.

Determination of sialic acid. Warren's (1959) method was used for the determinations. A synthetic crystalline preparation of *N*-acetylneuraminic acid obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) (type IV, lot no. 106B-0740) was used as a standard in the determinations; this material was stated by the suppliers to have a purity of 99+% by chromatographic methods, and infrared analysis showed a spectrum that conformed to the structure of *N*-acetylneuraminic acid. The compound had m.p. 186° (corr.). Samples (78 μg.) of the commercial product were homogeneous when examined by descending paper chromatography on Whatman no. 1 chromatography paper with butan-1-ol–propan-1-ol–0·1 N-HCl (1:2:1, by vol.) as solvent. Before use the chromatography paper was washed with 0·1 N-HCl followed by copious washings in water and then air-dried. The chromatograms were developed by dipping in the orcinol–trichloroacetic acid reagent of Klevstrand & Nordal (1950), after which they were heated at 105° for 10 min. Quantities (56 μg.) of authentic *N*-acetylneuraminic acid isolated from the mucin of sheep submaxillary gland (kindly given by Professor G. Blix, Uppsala, Sweden) were used as standards in the chromatography. The synthetic and natural compounds possessed identical R_F values in the above solvent.

The chromophore produced in the assay was extracted into cyclohexanone (British Drug Houses Ltd.); this solvent was redistilled immediately before use and the fraction boiling at 154–156° collected. Recrystallization from hot water of the 2-thiobarbituric acid, as described by Warren (1959), did not give better determinations than samples not so treated.

Mouse strains. Two strains of mice were used. (1) An inbred strain of an AK line, obtained 15 years ago from the Chester Beatty Research Institute, London, susceptible to leukaemia, provided the bulk of the normal and leukaemic cells. (2) About 13 years ago a sub-line was derived from this strain that is resistant to leukaemia when implanted with leukaemic cells; this sub-line provided the normal lymph-node cells of the resistant type.

Preparation of cell suspensions. (*a*) Normal lymph cells. Immediately after a mouse was killed by cervical dislocation it was pinned out in a supine position and the skin was cut in a median incision extending from the pubic to the neck region. The cut was extended over the upper part of both arms and legs and the two large skin flaps were pinned out under slight tension. This allowed for a quick dissection of both inguinal and axillary lymph nodes and avoided contamination of the nodes with blood. The nodes were carefully freed from surrounding adipose and connective tissue in Petri dishes standing on crushed ice; they were held in position by their connective tissue and care was taken not to squeeze them with the instruments. The thin connective-tissue capsule of the nodes freed from the surrounding tissue appeared smooth and shiny. About 40–80 nodes were thus prepared for each experiment. If in any mouse a lymph node was either enlarged or slightly red instead of having the normal grey-glassy appearance, the animal was discarded, but this was rare. The nodes were collected in a small covered container standing in crushed ice, and were minced with a fine sharp pair of scissors to form a homogeneous pulp; this was transferred to a universal container standing in crushed ice, and ice-cold Tyrode solution, containing 0·1% glucose, was added in the ratio of approx. 1 part wet wt. of minced lymph nodes to 20 vol. of Tyrode solution. The bottles were shaken gently for a few minutes, which produced a milky suspension, and then were left for about 1 min. to permit the larger fragments to settle. The supernatant suspension was transferred with a wide bore pipette into a cold centrifuge tube for washing. These suspensions contained 90–95% lymphocytes, 2–4% lymphoblasts, 1–3% monocytes and reticulum cells and 1–3% erythrocytes.

(*b*) Leukaemic cells. This acute lymphoblastic leukaemia is transmitted by implanting a small fragment of leukaemic tissue subcutaneously; the leukaemic cells spread rapidly throughout the organism and infiltrate almost all organs including bone marrow, lymph nodes and blood. The life-span of the mice after implantation of the leukaemic tissue is about 3 weeks. At the site of the original implant (in the right flank) a nodule is formed that is composed almost exclusively of leukaemic lymphoblasts. During the second week this nodule can be dissected out and provides about 0·1 g. of leukaemic tissue. The nodules of several mice were collected in a small container standing in crushed ice, and were then cut into small fragments with a fine pair of scissors until they formed a homogeneous-looking pulp; this was transferred to ice-cold Tyrode solution containing 0·1% glucose (1 part wet wt. of leukaemic cells to about 20 vol. of Tyrode solution). The bottle was shaken for a few minutes and left for about 1 min., and the milky supernatant was pipetted into another bottle. Care was taken to keep the cells cool throughout the procedure, and sterile technique, glassware and solutions were used.

Recently the leukaemic tissue was put through a Craigie (1949) pressure mincer to obtain a homogeneous suspension of leukaemic cells; this was labour-saving when larger quantities of leukaemic tissue were available. Those parts of the mincer (constructed by Mr C. E. Mallows, Strangeways Research Laboratory) that came into contact with biological material were made from stainless steel 18/8. The main screw ($\frac{3}{8}$ in. × 20 threads/in.), for forcing the graded plungers down the barrel of the mincer, was turned at the end to engage with the plungers and passed through a threaded cap made of aluminium-alloy rod [Blackburns (London) Ltd., London, W.C. 1, specn. BL7]. The plungers had 12 equally spaced V-cuts, made with a 90° included-angle cutter, on a diameter of $\frac{5}{8}$ in. The depth of the cuts on the coarse plunger was 1·0 mm. and that of those on the fine plunger 0·5 mm. The aluminium-alloy collar overcame the risk of the apparatus becoming seized up, as is likely with an all-steel instrument. In this work, satisfactory suspensions could be obtained with the fine plunger alone.

The cells, whether normal or leukaemic, were then separated from their ambient fluid by centrifugation at 500*g* for 10 min., resuspended in physiological saline with a ratio of medium to cells of at least 50:1, and again centrifuged. All cells were washed three times in physiological saline, and are termed 'standard washed cells'.

Treatment of cells with neuraminidase. Standard washed cells were suspended in 0·145 M-NaCl made 5 mM with respect to $CaCl_2$, to which neuraminidase solution was added to a final enzyme concentration of 20 units/ml. of solution (enzyme/cell ratios 20 units/$2·5 \times 10^8$ leukaemic cells and 20 units/$6·2 \times 10^8$ non-leukaemic cells). To maintain the pH at $7·2 \pm 0·2$ it was necessary to make the physiological saline 2 mM with respect to $NaHCO_3$. Under these conditions neuraminidase possessed 60% of its activity as measured by the release of sialic acid (Warren, 1959) from human glycoprotein fraction VI (batch no. K2201; Mann Laboratories Inc., New York, N.Y., U.S.A.), as compared with that released from the same substrate at the enzyme's pH optimum of 5·5. Equivalent quantities of cells were incubated in the above medium without added enzyme or containing an appropriate amount of heat-inactivated (100° for 45 min.) neuraminidase. Samples of such suspending medium were either made 0·1 N with respect to H_2SO_4 and kept at 90° for 1 hr., or treated with appropriate amounts of neuraminidase (Simon-Reuss *et al.* 1964) before microchemical analysis (Warren, 1959). An optimum time of 1 hr. for incubation of the cells with neuraminidase at 37° was chosen, as the action of the enzyme was then complete when determined by electrophoretic measurements. Treated cells were centrifuged at 500*g* for 10 min. and the supernatant fluid was quantitatively removed with a Pasteur pipette, the tip of which had been suitably drawn out. These cells were then washed three times in physiological saline as described above.

Fixation of cells. Standard washed cells were treated with aq. 2% (w/v) acetaldehyde or aq. 1·5% (w/v) formaldehyde under the conditions developed by Heard & Seaman (1961) for erythrocytes and subsequently used by Simon-Reuss *et al.* (1964) for various mammalian tissue cells. In addition some samples of neuraminidase-treated cells were fixed with 2% (w/v) acetaldehyde after three washes in physiological saline; the fixed cells were washed three times in physiological saline. Smears of a number of samples were made and stained with May–Grunwald and Giemsa dyes, and a differential count was recorded.

Electrophoretic mobilities. Measurements were made at $25 \pm 0·1°$ in a horizontal cylindrical chamber of small volume equipped with reversible Ag–AgCl electrodes (Seaman & Heard, 1961). The chamber was viewed by transillumination in the apparatus described by Bangham, Flemans, Heard & Seaman (1958) and obtained from Rank Bros., Bottisham, Cambs. Some preliminary measurements were also made in a cylindrical chamber equipped with grey platinum electrodes (Bangham *et al.* 1958). The mobilities of the cells were calculated in μ/sec./v/cm.; each value was obtained by timing the movements of at least 10 cells with reversal of polarity after each measurement. In this work the setting-up and operating procedures adopted were identical with those described in detail elsewhere (Bangham *et al.* 1958; Seaman & Heard, 1961; Seaman, 1965). The alignment of the apparatus was checked by the determination of the mobility of washed human erythrocytes in 14·5 mM-NaCl in 4·5% (w/v) sorbitol made 0·3 mM with respect to $NaHCO_3$, and in physiological saline (Heard & Seaman, 1960; Seaman & Heard, 1961); normal blood for this purpose was obtained from healthy donors of the phenotype ORh+ and taken into acid citrate–dextrose medium under standard National Blood Transfusion Service conditions. Specimens were stored at 4° and used up to 5 days after collection.

Electrophoretic mobilities of cells in sorbitol–saline media were corrected to the viscosity of standard saline at 25° (Simon-Reuss *et al.* 1964).

Treatment of cells with diazomethane. Standard washed aldehyde-fixed cells were washed three times in water, once in 0·05 N-HCl and twice in ethanol–diethyl ether (5:1, v/v); finally they were treated at 37° for 1 hr. with excess of diazomethane in diethyl ether, diluted 1:2 (v/v) with ethanol, as used for human erythrocytes (Seaman & Cook, 1965). Treated cells were subsequently washed as in the above procedure but in reverse order, and without the wash in HCl. As controls, aldehyde-fixed leukaemic cells and normal lymph cells were submitted to the above procedure but without diazomethane; they showed no significant change in electrophoretic mobility when measured in physiological saline.

Viability test with Erythrosin B. The method of Phillips & Terryberry (1957), who used Erythrosin B in viability tests on cells growing *in vitro*, was found to be suitable. In a suspension of standard washed leukaemic cells in physiological saline, 12% of the population took up this dye; 19% of the cells in the suspension incubated at 37° for 1 hr. with and without neuraminidase and 20% of the cells in the controls were stained. Thus under these conditions the enzyme does not kill the cells. Electrophoretic measurements made on cells from a fresh suspension, containing approx. 12% of dead or dying cells, gave the same values as those obtained from suspensions in which the dead cells were increased by some 8% as in the above experiment.

RESULTS

The present results were obtained over a period of almost 2 years. During this time cells from many groups of mice from the same lines were prepared for the experiments, but no variations in the behaviour of the cell population of either the normal lymph node or of their leukaemic counterparts were noted. The readings obtained remained constant whether the electrophoretic mobility was measured with platinum electrodes or with silver–silver chloride electrodes; the latter had the practical advantage that a small electrophoretic cell was used in which the volume required for measurements was only one-fifth of that needed for the electrophoretic cell with platinum electrodes. The mean electrophoretic mobilities, with S.D., for the various cell types and their isoelectric points are listed in Table 1.

Electrophoretic mobility of untreated cells. The cells of normal lymph nodes, from mice either susceptible or resistant to leukaemia, had an electrophoretic mobility at pH 7·2 of $-1·27 \pm 0·03$ (8) μ/sec./v/cm. and $-1·29 \pm 0·01$ (4) μ/sec./v/cm.

Table 1. *Electrokinetic parameters of leukaemic and non-leukaemic cells and the sialic acid released from them by neuraminidase*

Numbers in parentheses refer to the numbers of experiments, each consisting of at least ten observations. The electrophoretic mobility of the cells was measured in physiological saline kept at 25±0·1°. For details of the saline and of experimental methods, see the text.

Type of cell	Electrophoretic mobility in physiological saline (μ/sec./v/cm. at pH 7·2±0·2)			Estimated isoelectric point of untreated cells	Sialic acid (expressed as μmole of *N*-acetyl-neuraminic acid) released from 10^8 cells
	No treatment	Aldehyde treatment	Neuraminidase treatment		
Normal lymph-node cells (susceptible to leukaemia)	−1·27±0·03 (8)	−1·29±0·04 (5)		<2·2	
Normal lymph-node cells (susceptible to leukaemia) after 1 hr. at 37°	−1·32		−0·94		
Normal lymph-node cells (resistant strain)	−1·29±0·01 (4)				
Formaldehyde-fixed lymph-node cells (resistant strain) after 1 hr. at 37°		−1·28	−0·96		
Leukaemic cells	−1·04±0·06 (21)*	−1·28±0·06 (12)		3·3	
Leukaemic cells after 1 hr. at 37°	−1·08 −1·10 (−1·27)‡		−0·53; −0·54† −0·58 (−1·01)‡		
Aldehyde-fixed leukaemic cells		−1·28±0·06 (12)	−1·01±0·07 (7)		0·0093±0·0033 (4)

* Leukaemic cells in medium of low ionic strength (0·0145 g.ion/l.), pH 7·2±0·2, have a mobility of −2·07±0·06 (5) μ/sec./v/cm. and an estimated isoelectric point at pH 3·7.

† Duplicate determinations on same batch of cells.

‡ Numbers in parentheses represent the electrophoretic mobility of samples after fixation in acetaldehyde.

respectively. In contrast, the mobility of the leukaemic cells was only −1·04±0·06 (21) μ/sec./v/cm.

Effect of lower aldehydes on electrophoretic mobility. Both acetaldehyde and formaldehyde gave similar results. Normal lymph cells showed no significant changes from the untreated controls, −1·29±0·04 (5) μ/sec./v/cm. for the aldehyde-treated normal cells and −1·27±0·03 μ/sec./v/cm. for the untreated controls; there was no difference in the values, whether the normal cells came from mice susceptible or resistant to leukaemia. In contrast, the electrophoretic mobility of the aldehyde-treated leukaemic cells rose from −1·04±0·06 (21) to −1·28±0·06 (12) μ/sec./v/cm.; thus aldehyde treatment brought the value for the leukaemic cells to that for normal lymph cells, whether aldehyde-treated or untreated.

Effect of incubation with neuraminidase. Exposure of untreated or aldehyde-fixed normal lymph cells to this enzyme lowered their electrophoretic mobility by about 0·35 to −0·95 μ/sec./v/cm., a decrease of 27%. The electrophoretic mobility of leukaemic cells after incubation with neuraminidase was lowered by 0·54 to −0·55 μ/sec./v/cm., representing a decrease of 49%. The isoelectric point of the treated cells was raised to about pH 4.

When aldehyde-treated leukaemic cells (electrophoretic mobility −1·28 μ/sec./v/cm.) were incubated with neuraminidase their mobility decreased by just over 21% to 1·01±0·07 (7) μ/sec./v/cm. Under these circumstances the amount of sialic acid in the supernatant fluid of 10^8 aldehyde-fixed leukaemic cells was 0·0093±0·0033 (4) μmole, calculated as *N*-acetylneuraminic acid; in contrast, under the same conditions, the same number of normal lymph cells yielded a concentration of sialic acid in the supernatant too low to be determined.

When the order of treatment of leukaemic cells with aldehyde and neuraminidase was reversed, the same values for their electrophoretic mobility were obtained; this indicated that the sites on which the two reagents operate were not closely connected and that the decrease in mobility after neuraminidase treatment was not due to the generation of cationic groups.

Treatment with diazomethane. The electrophoretic mobility of aldehyde-stabilized normal lymphocytes

Table 2. *Reversible response of standard washed cells to temporary changes in their environment*

Standard washed leukaemic and non-leukaemic cells were suspended either in medium of low ionic strength for 5 min. or in aq. 0·145 M-NaCl at 25° and maintained for 5 min. at various pH values; they were then returned to either physiological saline or pH 7.

	Initial mobility (μ/sec./v/cm. at pH 7)	pH for reversal	Ionic strength for reversal (g.ion/l.)	Final mobility (μ/sec./v/cm. at pH 7)
Leukaemic cells	−1·00	2·7	—	−1·12
	−1·05	3·9	—	−1·08
	−1·05	10·1	—	−1·03
	−1·00	—	0·0145	−0·98
Normal lymph cells (from the susceptible strain of mice)	−1·28	3·0	—	−1·30
	−1·32	9·5	—	−1·30

and leukaemic cells was decreased to zero when the cells were treated with diazomethane. The same result was obtained when neuraminidase treatment was interposed between the aldehyde fixation and exposure to diazomethane. This decrease of the electrophoretic mobility to zero indicates that fortuitous ion-binding on the cell surface cannot be the cause of the charge remaining after either stabilization by aldehydes alone or subsequent treatment with neuraminidase.

There were no significant differences in electrophoretic mobility in physiological saline between control batches of aldehyde-fixed normal lymphocytes, and leukaemic cells that had not been submitted to diazomethane but exposed to the solvent used for the diazomethane (diethyl ether–ethanol, 1:5, v/v) and to the washings in 0·05 N-hydrochloric acid. The value for leukaemic cells was −1·27 μ/sec./v/cm. and that for normal lymphocytes −1·28 μ/sec./v/cm.

Reversibility of the electrophoretic mobility. Table 2 lists the observations made on untreated normal and leukaemic cells before and 5 min. after exposure to high or low pH; both sets of measurements were made on cells suspended in aq. 0·145 M-sodium chloride at 25°. It can be seen that surface changes of both normal and leukaemic cells were reversible under these conditions, and that exposure of leukaemic cells to a medium of low ionic strength did not cause irreversible changes in their electrophoretic mobility. These observations indicated a considerable stability of the surface layer of both normal and malignant lymph cells.

pH–*mobility relationships.* Normal lymph-node cells from mice susceptible or resistant to leukaemia, whether untreated or fixed in formaldehyde or acetaldehyde, gave similar results when their electrophoretic mobilities were determined in aq. 0·145 M-sodium chloride over the pH range 2·7–9·1. These observations are depicted in Fig. 1, which also shows the lowered electrophoretic mobilities of normal cells after neuraminidase treatment. Cells

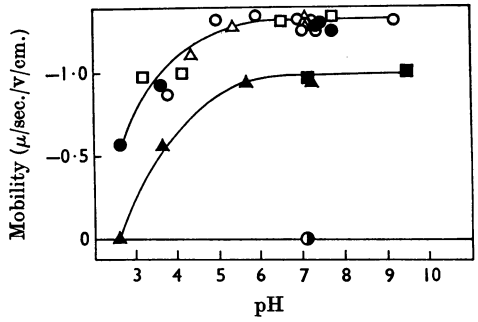

Fig. 1. pH–mobility relationships after various treatments of standard washed normal lymph-node cells suspended in aq. 0·145 M-NaCl at 25°. The treatment conditions and details of the mobility determinations are given in the Materials and Methods section. Mobility values for untreated cells from susceptible mice (○), untreated cells from resistant mice (△), and formaldehyde-fixed cells (□) and acetaldehyde-fixed cells (●) from susceptible mice form the upper curve. Neuraminidase-treated cells from susceptible mice (▲) and formaldehyde-fixed cells from resistant mice treated with this enzyme (■) have mobilities falling on the lower curve. ◑, Mobility value of neuraminidase-treated formaldehyde-fixed cells from resistant mice after diazomethane treatment.

from susceptible and resistant mice again gave similarly decreased values. The electrophoretic mobility of aldehyde-fixed neuraminidase-treated cells, exposed to diazomethane, was decreased to zero.

Leukaemic lymphoblasts were measured under the same conditions; Fig. 2 shows the values of the electrophoretic mobilities of untreated leukaemic cells over the pH range 2·7–10·3. All values were below those for normal cells, as can be seen by comparing the curves marked ○ in Figs. 1 and 2. In contrast, after either formaldehyde or acetaldehyde fixation, leukaemic cells had an increased

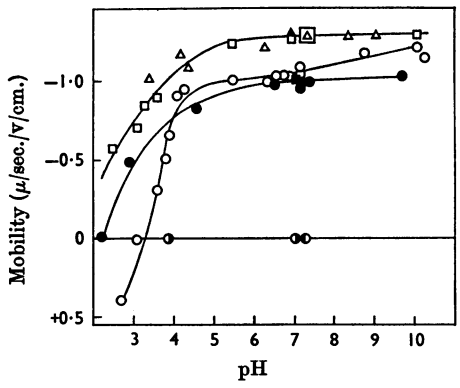

Fig. 2. pH–mobility relationships after various treatments of standard washed leukaemic cells suspended in aq. 0·145 M-NaCl at 25°. The treatment conditions and details of the mobility determinations are given in the Materials and Methods section. ○, Mobility values for untreated cells. Formaldehyde-fixed (△) and acetaldehyde-fixed (□) cells have mobility values falling on the upper curve. ●, Mobilities of aldehyde-fixed cells on treatment with neuraminidase. Mobility values for a sample of leukaemic cells incubated for 1 hr. at 37° with (■) and without (▲) neuraminidase followed by acetaldehyde fixation, as well as formaldehyde-fixed cells treated with diazomethane (◑) and neuraminidase-treated formaldehyde-fixed cells treated with diazomethane (◐), are indicated.

electrophoretic mobility over the whole pH range 2·5–10·1 and the values approached those obtained for aldehyde-stabilized normal lymphocytes.

When aldehyde-stabilized leukaemic cells were treated with neuraminidase, their initially higher mobility was lowered in comparable proportion over the pH range investigated. Exposure of aldehyde-fixed cells to diazomethane removed their electrophoretic mobility at acid pH. This was not checked at alkaline pH because ester hydrolysis occurs above pH 7·5 (Gittens & James, 1963).

Cytological structure. Smears of the cell suspensions used in these experiments and stained with May–Grunwald and Giemsa stains were examined microscopically in representative samples of untreated acetaldehyde- and formaldehyde-fixed leukaemic cells, and of untreated and formaldehyde-fixed lymph cells. The cells treated with acetaldehyde developed an orange colouration of intracellular origin that has been noted in other cells during fixation (Cook *et al.* 1962). In preparations from leukaemic cell suspensions, 97·5–99·3% of the cells were leukaemic, the remaining 0·7–2·5% cells being erythrocytes, whereas suspensions of normal lymph nodes contained approx. 90% lymphocytes and 3–8% lymphoblasts, monocytes and reticulum cells; the remaining cells were erythrocytes. No major problems of heterogeneity therefore arose to complicate the determinations.

Aldehyde fixation was shown microscopically not to alter drastically the cells' morphology; the shape of nuclei and nucleoli was well preserved, though the cytoplasm had lost almost all basophilia.

DISCUSSION

The electrophoretic properties of biological cells are due mainly to the presence of ionogenic groups at their peripheries. Hence changes in a cell's electrophoretic mobility caused by specific chemical reagents or enzymes under suitably controlled conditions permit certain deductions to be drawn about the chemical nature of the peripheral regions of the plasma membrane. Undoubtedly the electrophoretic characteristics of a cell depend on the organ, the site of origin and possibly the state of division of the nucleus and cytoplasm. Although the presence of sialic acids at the surface of most animal cells so far examined has been demonstrated and shown to be an important ionogenic determinant, the role of these acids in the glycoproteins of cell membrane has not yet been fully elucidated (Simon-Reuss *et al.* 1964).

It has been pointed out (Cook *et al.* 1962) that results obtained from cells suspended in simple electrolytes should be related to behaviour *in vivo* with extreme caution. That the present findings might be complicated by contamination of the cell surface by cell debris, although not completely excluded, appears unlikely as there was no evidence of bimodal distribution of mobilities, and moreover identical results were obtained whether cells were prepared by chopping with fine scissors or by using a Craigie mincer. It is suggested that the present results, which are reproducible with respect to time and reversible with changes in the environmental conditions, represent definite alterations in the physical properties of the surface membranes. In the leukaemic cells the isoelectric point measured in 0·145 M- and 14·5 mM-sodium chloride was only slightly affected by the ionic strength, which suggests that products of cell leakage, if they are adsorbed on the surface, exert little influence on the electrophoretic properties of the cell under the experimental conditions employed.

It can be seen (Table 1) that the electrophoretic properties of the leukaemic cells differ in several respects from those of normal lymph cells. The mobility of untreated leukaemic cells in physiological saline ($-1{\cdot}04$ μ/sec./V/cm.) is significantly lower than the mobility of normal lymph cells, most of which are lymphocytes ($P < 0{\cdot}001$ with 27 degrees of freedom). Where possible the mobility of normal

lymphoblasts, which are larger than lymphocytes, was recorded; the former represent a small minority of the cellular population and it is not possible to obtain sufficient readings at the stationary level in the chamber to deduce a mean value. The migration times of those lymphoblasts that were timed, however, fell within the lymphocyte transit values, which was not true of the occasional erythrocyte observed in the field of vision. No significant difference could be detected between normal lymph-node cells derived from mice resistant and susceptible to leukaemia.

Lower aldehydes in aqueous media at neutral pH react mainly with potential cationogenic groups and therefore the reaction is confined mainly to cellular proteins. In addition, cross-linking of cellular proteins by aldehydes greatly increases the stability of a cell to reagents that require non-physiological conditions (Heard & Seaman, 1961).

On treatment with aldehyde (Simon-Reuss *et al.* 1964) the leukaemic cells show a significant increase ($P < 0.001$ with 31 degrees of freedom) in their anodic mobility, indicating the presence of positive charges at the slip plane from potential $-NH_3^+$ groups that are eliminated by aldehyde (Heard & Seaman, 1961). This treatment, which causes a mobility equivalent to that of the normal cells, besides indicating the presence of cationic groups in the ultrastructure of the cell surface greatly increases the cells' electrokinetic stability; since many of the most useful reagents for characterizing ionogenic groups (e.g. diazomethane) require non-physiological conditions for their reaction, aldehyde-fixed material has additional advantages for this investigation.

Both types of cell must possess significant quantities of sialic acids at their peripheries, since neuraminidase causes a marked fall in electrophoretic mobility and free sialic acids (expressed as *N*-acetylneuraminic acid) can be demonstrated chemically in the supernatant fluid of treated leukaemic cells. There was no further decrease in electrophoretic mobility when such cells were re-exposed to a fresh solution of neuraminidase, and no sialic acid could be detected in the supernatant of this second incubation period.

Incubation of cells with neuraminidase at physiological pH has been used for erythrocytes (Cook *et al.* 1961) and for various other cell types (Simon-Reuss *et al.* 1964). Kraemer (1966), using a line of ovary cells of Chinese hamster, showed that the enzyme was sufficiently active at pH 7·0 to remove over 90% of the sialic acid from the cell surface, though the pH optimum is at 5·5. His tests with trypan blue and ours with Erythrosin B (Phillips & Terryberry, 1957) revealed no appreciable damage to cells by the enzyme at pH 7·0. It may be mentioned here that heat treatment at 40° for 2½ hr. does not affect the electrophoretic mobility of 6C3HED lymphosarcoma cells (Lowick, Purdom, James & Ambrose, 1961).

The decrease in electrophoretic mobility in response to neuraminidase appears to be due to the removal of the carboxyl groups of sialic acid. As this enzyme is an α-glycosidase it is unlikely to generate any cationic groups. The fact that there is a plateau in mobility over the pH range 5·8–9·4 for normal lymph cells treated with neuraminidase, and a similar plateau over the pH range 6–9·7 for aldehyde-fixed leukaemic cells incubated with this enzyme, further supports the view that no cationic groups were generated on the cell surface by exposure to neuraminidase.

It is unlikely that the enzyme binds to the cell surface to any extent. Heat-inactivated enzyme does not alter the electrophoretic mobility of the control cells and the decrease in mobility associated with neuraminidase treatment cannot be explained by adsorption, as this would cause an almost instantaneous change of mobility after the addition of the enzyme to the cell suspension.

Exposure of neuraminidase-treated leukaemic cells to acetaldehyde changes their mobility to that of cells first fixed with aldehyde and then treated with neuraminidase. With the non-malignant cells, neuraminidase causes a decrease in mobility of the same magnitude irrespective of whether the cells are normal and untreated or have been fixed previously with formaldehyde. Thus the order of exposure to the enzyme and the aldehyde makes little difference in the final effect on the electrophoretic mobility of either normal or leukaemic cells. This result not only confirms that neuraminidase treatment does not cause the production of any cationic groups, but also suggests that the removal of sialic acid in the unfixed cell takes place in the absence of any reorientation in the membrane with the appearance of new negative charges at the hydrodynamic plane of shear. The latter mechanism has been suggested by Wallach & Esandi (1964) to explain the lack of change in electrophoretic mobility after enzymic removal of sialic acid from MC1M tumours and sarcoma 37 (Cook, Seaman & Weiss, 1963).

In addition to treatment with neuraminidase and aldehydes, acid-washed aldehyde-treated cells were chemically modified with diazomethane, which reacts readily with carboxyl groups to produce esters. Treatment of an ethanolic suspension of *Aerobacter aerogenes* (Gittens & James, 1963) or aldehyde-stabilized human erythrocytes (Seaman & Cook, 1965) with diazomethane decreased their mobility to zero over a pH range where no hydrolysis of ester groups occurs. A similar result was obtained in the present investigation. Diazomethane, in addition to reacting with the carboxyl group of sialic acid, reacts with the remaining groups producing a cell with zero mobility. This fact

suggests that the residual charge remaining on the plasma membrane after aldehyde and neuraminidase treatment may be due to carboxyl groups, such as the β- and γ-carboxyl groups of aspartic acid and glutamic acid. The presence of these acids has been demonstrated in surface glycoproteins of erythrocytes (Cook, 1962; Cook & Eylar, 1965).

The mobility values obtained for standard washed leukaemic cells after reversible changes in environmental conditions (Table 2) fit the plateau region in the pH range 5–7·5 in the normal untreated-cell pH–mobility relationship (Fig. 2). A similar result is obtained for cells derived from normal lymph nodes of non-leukaemic mice. It is suggested therefore that the chemical structure responsible for the electrophoretic behaviour of these cells is stable under the experimental conditions used. Consequently the pH–mobility relationships can be used to obtain reliable information about the pK of ionogenic groups on the cell surface.

As shown in Fig. 2, the aldehyde-fixed cell shows about a 25% increase in mobility over the pH range 5·5–8·5 compared with the untreated leukaemic cell; as indicated above, this result is consistent with the presence of cationic groups, which is confirmed by the increase in the mobility of the cells at about pH 10 and the net positive mobility below pH 3·2. The positive groups, probably represented by amino groups, have pK about 10. However, above pH 10 the unfixed cell is liable to fairly rapid solubilization and measurements in excess of this value should be treated with extreme caution; similar caution must be exercised at the acid end of the pH–mobility relationship. The untreated and aldehyde-fixed normal lymphocytes from susceptible and resistant mice show the same pH–mobility relationship.

The electrokinetic behaviour of the leukaemic cells seems to arise from three ionogenic systems, two dissociable acid functions and one dissociable basic function: (1) a carboxyl group of a sialic acid, pK about 2·7; (2) an unidentified acidic group(s) of pK about 3·0–4·5; this is probably a β- or γ-carboxyl group derived from membrane protein to judge from the ease with which this group is methylated by diazomethane; (3) a basic group of pK about 10. The normal lymph cells seem to have similar acidic ionizable functions but to lack the basic group of pK about 10.

The precursor cell of the normal lymphocyte is the normal lymphoblast, which remains within the cellular network (the reticulum) of lymph nodes and the white pulp of the spleen. It proliferates there and nowhere else, and undergoes maturation to the normal lymphocyte that may be swept out into circulation, but does not proliferate under normal circumstances outside the lymph nodes and spleen. In contrast, the malignant precursor cell, the leukaemic lymphoblast of the acute leukaemia investigated in the present experiments, proliferates not only in lymph nodes and spleen but can leave these organs, migrate into the surrounding tissues or enter the circulation to settle down and proliferate anywhere else in the body as a truly invasive cell; it does not undergo maturation to a lymphocyte. Whether the difference in ultrastructure of the cell surface of the normal cell and of its malignant counterpart, as described in the present paper, has any direct bearing on the different behaviour of the two cell types remains a matter for further investigation.

It is probable that the present results mainly represent differences in the chemistry of the membrane sialoglycoproteins. It is noteworthy that the work of Gesner & Ginsburg (1964) suggests that the integrity of the sugars on rat lymphocytes enables these cells to traverse their unique route through the body, by acting as sites recognized by complementary structures on the surface of endothelial cells in the postcapillary venules of lymphoid tissue.

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