Biochimica et Biophysica Acta, 291 (1973) 107–115

BBA 76099

SURFACE PROPERTIES OF HUMAN LYMPHOCYTES

P. S. VASSAR, J. M. HARDS AND G. V. F. SEAMAN

Department of Pathology, Faculty of Medicine, University of British Columbia, Vancouver, B. C. (Canada) and Division of Neurology, University of Oregon Medical School, Portland, Oreg. 97201 (U.S.A.)

(Received July 10th, 1972)

SUMMARY

Cell electrophoresis of freshly isolated human lymphocytes revealed the following surface properties including (1) a positive branch of the pH mobility curve at low pH and, (2) an increase in negative charge after aldehyde treatment, indicating the presence of surface positive charges, (3) the presence of a relatively high surface pK of about 3.1 and, (4) the comparatively low surface concentration of N-acetylneuraminic acid, (5) lymphocytes separated from stored blood showed surface characteristics that deviated from fresh lymphocytes: the electrophoretic mobility of these cells was lower, not pH reversible and showed considerable variability.

INTRODUCTION

A series of recent publications has underscored the importance of the surface characteristics of lymphocytes in relation to their biological role and properties[1,2]. Peripheral blood lymphocytes suspended in media under physiological conditions display an anodic electrophoretic mobility[3,4]. Similar behavior has been observed for other blood elements including red blood cells[5], platelets[6] and polymorphonuclear leukocytes[7,8]. Working with rat lymphocytes, it has been shown that phytohemagglutinin interacts with the surface of the lymphocyte[9]. Lymphocytes are also involved in antigen–antibody reactions[10]. More recently, the human lymphocyte surface has been examined[3] and the observation made that different lymphocyte separation procedures did not affect the subsequently observed electrokinetic properties of the lymphocyte.

The sialyl residues of the peripheral zone of lymphocytes play a crucial role in the expression of antigen reactivity[11] and in the normal distribution of these cells in the body[12]. Woodruff and Gesner[12] have shown that neuraminidase-treated lymphocytes, when transfused into allogeneic or syngeneic recipients, are sequestered in macrophage pools rather than circulating as normal peripheral blood lymphocytes. Further work[13] has demonstrated that neuraminidase-treated lymphocytes have exposed antigenic sites and a susceptibility to the action of complement. It seems

clear, therefore, that a detailed knowledge of the molecular structure of the lymphocyte surface will produce a greater understanding of the mechanisms whereby the lymphocyte is able to carry out its vital biological functions.

MATERIALS

All compounds used were of analytical grade, and reagents were prepared in distilled water. Standard saline consisted of 0.15 M aqueous NaCl solution (pH adjusted to 7.2 ± 0.2, 285 ± 10 mosM/kg) by the dropwise addition of 0.5 M aqueous $NaHCO_3$ solution. Iso-osmotic phosphate-buffered saline, (pH 7.2 ± 0.1, 285 ± 10 mosM/kg) contained 50 parts of 0.15 M NaCl, 10 parts of 0.16 M NaH_2PO_4 and 40 parts of 0.13 M Na_2HPO_4. Formaldehyde (2 g/100 ml) was prepared by adding 2 g of paraformaldehyde to iso-osmotic phosphate-buffered saline and heating at 60 °C for 30 min as described by Pease[14]. Glutaraldehyde was obtained as a 70 g/100 ml aqueous solution stored under freon. It was sufficiently pure on the basis of spectral analysis to require no re-distillation. A 1.65 g/100 ml solution of glutaraldehyde was made up in iso-osmotic buffered saline.

Neuraminidase (Behringwerke) had an activity of 500 units/ml, where 1 unit of activity is that quantity of enzyme which would liberate 1 mg of sialic acid from a glyco-protein substrate in 15 min at 37 °C.

Blood was taken from normal healthy human volunteers under sterile conditions, in 500-ml units, using acid–citrate–dextrose as the anticoagulant (N.I.H. formula A).

METHODS

Lymphocyte isolation technique

The method of Agostoni and Idéo[15] was used. This technique uses NH_4Cl lysis of red blood cells followed by cotton wool column separation of lymphocytes from granulocytes and platelets. According to these authors, cell viability was excellent and the lymphocyte recovery was over 50%; our lymphocyte preparations were examined microscopically and showed 95–100% purity. They were also exposed to the trypan blue dye exclusion test and were found to have approx. 95% viability. There is some evidence that this method selects T cells preferentially[16,17]. Fresh lymphocytes were separated from blood within 1 h after collection from the donor. Stored lymphocytes were obtained from 500-ml units of blood which had been stored from 7 to 20 days at 4 °C in sterile plastic packs.

Neuraminidase treatment

Standard washed and centrifugally packed (150 × *g*, 3 min, 20 °C) normal or aldehyde-treated lymphocytes were treated with neuraminidase as follows: 4 vol. of neuraminidase reagent solution (50 units/ml neuraminidase in a solution of 0.15 M NaCl and 2 mM $CaCl_2$ adjusted to pH 6.6 by the dropwise addition of 0.5 M $NaHCO_3$) were mixed with 1 vol. of the lymphocyte suspension (approx. $5 \cdot 10^6$ lymphocytes per ml in standard saline) and the suspension incubated at 37 °C for 45 min. The neuraminidase-treated lymphocytes were then washed three times in standard saline.

Aldehyde fixation

Lymphocytes (1 vol., approx. $5 \cdot 10^7$ per ml) were added to the aldehyde fixative (9 vol.), mixed and allowed to fix for at least 36 h at 4 °C. The aldehyde solutions used were 2 g/100 ml formaldehyde in iso-osmotic phosphate buffer and 1.65 g/100 ml glutaraldehyde in iso-osmotic phosphate buffer. The aldehyde-treated lymphocytes were washed three times in standard saline prior to neuraminidase treatment and electrophoretic examination.

Measurement of cellular electrophoretic mobility

Electrophoretic mobility measurements were carried out in a cylindrical chamber apparatus at 25 °C as described previously[18]. The electrophoretic mobilities of the lymphocytes were expressed as $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ and were based on the times of migration of at least ten cells per run. Isotonic aqueous solutions of HCl or NaOH were added to standard saline for studies of variation of the electrophoretic mobility of cells with pH. The examination of the electrokinetic reversibility of the cells was conducted as reported previously[19].

Calculation of lymphocyte surface-charge density

The electrokinetic potentials (ζ) were calculated from the Helmholtz–Smoluchkowski equation:

$$\zeta = \frac{4\pi\eta U}{\varepsilon}$$

where η and ε are, respectively, the viscosity and dielectric constant of the solution in the surface phase and are assumed to be equal to the values for the bulk of the suspending medium. U is the electrophoretic mobility. The zeta potentials (mV) of most cells in standard saline are less than 25 mV and thus, to a reasonable degree of approximation, the surface charge densities (σ) of the cells may be computed by means of the Gouy–Chapman equation[19]. The correction factor α, proposed by Haydon[20] was neglected since no plausible value could be ascribed to it, but it should be noted that as a consequence the calculated surface-charge density could be in error by a factor of up to 2-fold.

In order to calculate the number of electron charges per lymphocyte an estimate of the mean surface area of the cells is required. Several estimates of lymphocyte volumes are available in the literature. We have calculated the surface area of the human lymphocyte to be approximately 200 μm^2 on the basis of the mean volume of lymphocytes reported in four recent publications (refs 21–24). Using the facts that $4.802 \cdot 10^{-10}$ esu is equivalent to one electron charge and that the mean surface area is 200 μm^2 the numbers of electron charges per lymphocyte have been estimated (Table I).

RESULTS

The electrokinetic properties of normal human lymphocytes, and lymphocytes modified by treatment with aldehydes and/or neuraminidase are presented in Table I. Electrokinetic charge densities and the number of electron charges per cell are also included. For purposes of comparison, data on human normal red blood cells and polymorphonuclear leukocytes are tabulated[7].

TABLE I

ELECTROKINETIC PARAMETERS FOR LYMPHOCYTES IN STANDARD SALINE AT pH 7.2 ± 0.2 AND 25 °C

Erythrocyte and polymorphonuclear leukocyte values are given for comparisons.

System	*Electrophoretic mobility* ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1} \pm S.D.$)		*Charge density* ($esu\ cm^{-2}$)		*Electron charges per cell* ($\cdot 10^7$)	
	Control	*Neuraminidase-treated*	*Control*	*Neuramidase-treated*	*Control*	*Neuraminidase-treated*
Fresh lymphocytes	−1.05 ± 0.03 (190)	−0.46 ± 0.04 (100)	3628	1575	1.50	0.65
Formaldehyde lymphocytes	−1.21 ± 0.06 (70)	−0.64 ± 0.07 (70)	4197	2196	1.73	0.91
Glutaraldehyde lymphocytes	−1.26 ± 0.04 (50)	−0.78 ± 0.10 (100)	4376	2682	1.80	1.11
Erythrocytes	−1.08 ± 0.03 (160)	−0.34 ± 0.04 (30)	3735	1163	1.27	0.40
Polymorphonuclear leukocytes	−0.90 ± 0.04 (170)	−0.36 ± 0.04 (40)	3101	1231	1.83	0.73

In Fig. 1, the electrophoretic behaviour of fresh untreated and neuraminidase-treated lymphocytes are compared over a range of pH. Approximately 45% of the surface negative charge of the fresh untreated lymphocyte is contributed by membrane-bound neuraminate ions. Although the lymphocyte surface is predominantly anionic in character, the positive branch to the pH *versus* mobility relationship at low pH, and the increase in the anodic mobility at high pH values, implies the presence of a significant number of positively charged surface groups.

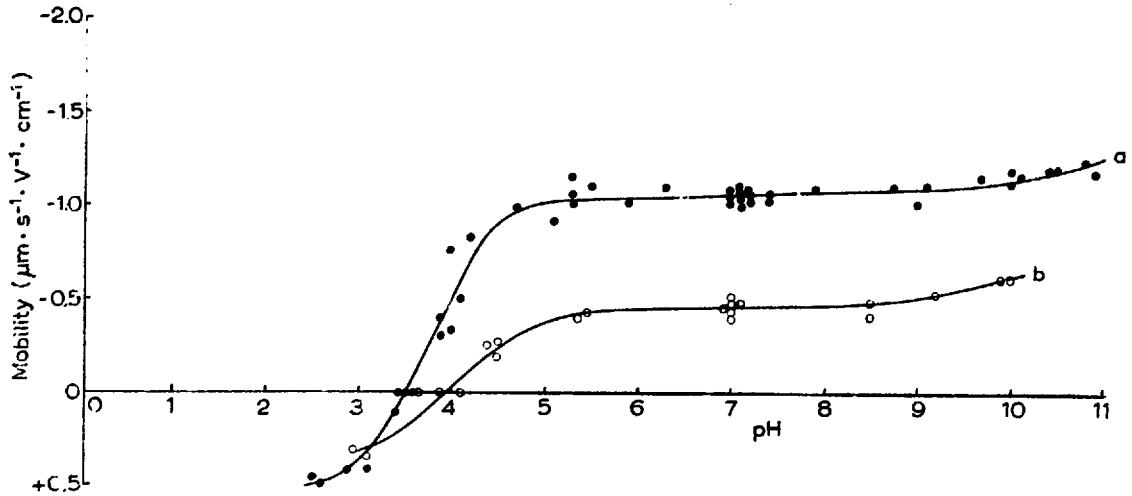

Fig. 1. pH *versus* electrophoretic mobility relationships for fresh and neuraminidase-treated human peripheral blood lymphocytes. Lymphocytes were suspended in standard NaCl at 25 °C; after the pH had been altered by the addition of 0.145 M HCl or 0.145 M NaOH and the mobilities measured, the pH was returned to 7.2 ± 0.2 to show that no irreversible changes had been produced by the alteration in pH. The effects of pH variation on the mobility of fresh lymphocytes is shown in a) and on the mobility of neuraminidase-treated lymplocytes in (b). The points have been accumulated from separate cell preparations.

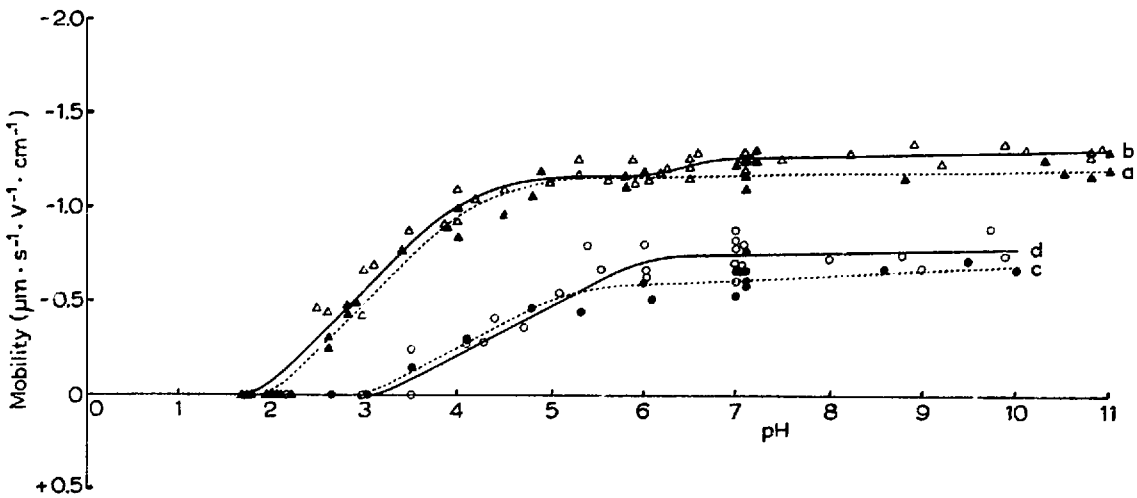

Fig. 2. pH *versus* electrophoretic mobility relationships for lymphocytes fixed in formaldehyde or glutaraldehyde, and for lymphocytes fixed in formaldehyde or glutaraldehyde and then treated with neuraminidase. The cells were fixed for at least 36 h before neuraminidase treatment and electrophoresis; the enzyme treatment was carried out immediately prior to electrophoresis. Curve (a) shows pH mobility changes for formaldehyde-fixed lymphocytes, (b) for glutaraldehyde-fixed lymphocytes, (c) for formaldehyde-fixed and neuraminidase-treated lymphocytes and (d) for glutaraldehyde-fixed and neuraminidase-treated lymphocytes. Points have again been accumulated from several separations.

Fig. 2 depicts the influence of formaldehyde and glutaraldehyde fixation on the pH *versus* mobility relationship of the human lymphocyte. The figure also shows the effect of treating these fixed cells with neuraminidase. At pH 6 it may be seen that aldehyde blocking of surface groups, probably amino groups, results in approximately a 10% increase in surface negative charge; there is also, as expected, a concomitant disappearance of the positive branch to the pH *versus* mobility relationship. At pH values above 6.5, there is an additional increase in mobility of glutaraldehyde-fixed cells such that they then possess about a 10% higher mobility than the corresponding formaldehyde-fixed cells. The observed differences between glutaraldehyde-fixed cells and cells treated with monoaldehydes has been documented already for other cell systems[25]. The pK of anionic groups, half the mobility of the plateau value, is estimated to be approximately 3.2 and the pK of the groups remaining after removal of neuraminate ions is judged to be approximately 4.3, consistent with the presence of other protein carboxyl groups.

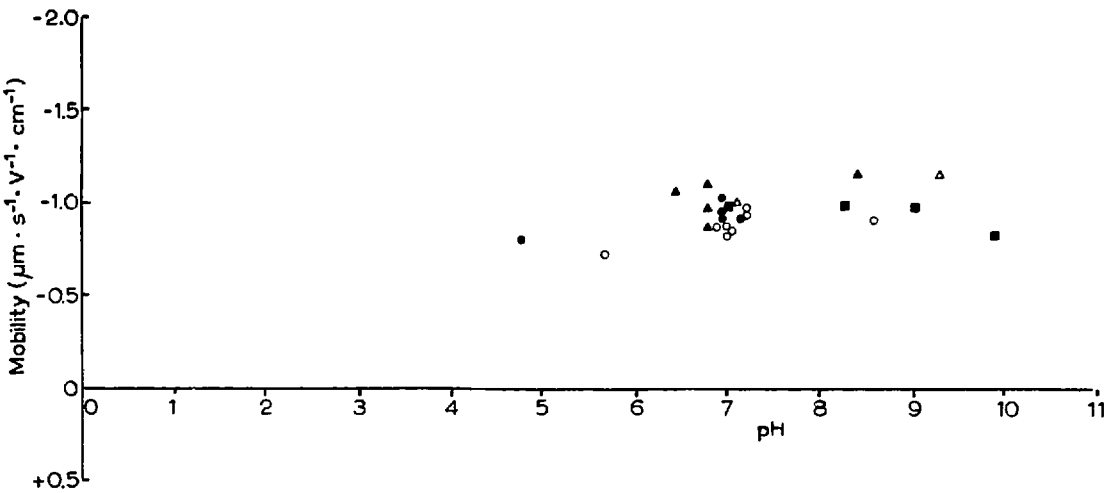

Fig. 3. pH *versus* electrophoretic mobility relationships for stored untreated human peripheral blood lymphocytes. The results have been accumulated from 5 separations, and the electrophoretic mobilities were measured immediately following separation.

In marked contrast to the findings with fresh lymphocytes and fresh lymphocytes which have been modified by aldehydes and/or neuraminidase, Fig. 3 summarizes the results obtained with stored lymphocytes. The mobility values at neutral pH are lower, variable and are not pH reversible.

DISCUSSION

Evidence in the literature suggests that human peripheral blood lymphocytes comprise at least two populations designated as bursa-derived (B) lymphocytes and thymus-derived (T) cells[16]. These two classes of lymphocytes have apparently been separated by means of free flow electrophoresis[26]. One lymphocyte population obtained from a spleen-mediated, graft host, reaction was found to possess a higher electrophoretic mobility than the associated antibody-forming cells[26]. In the present study there appeared to be only one population of lymphocytes by electrokinetic criteria. However, the method used for the isolation of the lymphocytes is believed to select the T cells preferentially[16,17]. There is some evidence to suggest that

exposure of cells to polymeric materials such as gelatin[3] or ficoll–hypaque[27] may under certain experimental conditions, result in their irreversible surface adsorption, thus resulting in a modification in subsequent cell electrokinetic properties[28]. There is no doubt, however, that whatever the cell separation technique employed, the lymphocytes should be essentially free of any other blood elements since it is difficult to distinguish readily between lymphocytes and some other cell types when observed in free suspension at the stationary level in the electrophoresis chamber.

The surface of the human peripheral blood lymphocyte differs appreciably from that of either the red blood cell or polymorphonuclear leukocyte both in ionogenic complexity and in having an appreciably lower content of surface sialic acid. At least five different types of charged groups have been identified at the surface of lymphocytes[2]. We have found that only 45% of the net negative charge of the lymphocyte arises from membrane-bound sialyl residues, (Fig. 1), whereas 62% of the charge of the red blood cell and 60% of the charge of polymorphonuclear leukocytes arise from this source. The complexity of the lymphocyte surface is perhaps a reflection of the diverse biological functions undertaken by such cells.

It is seen from Fig. 1 and Table I that the mean electrophoretic mobility for fresh normal human lymphocytes in standard saline at pH 7.2 ± 0.2 is $-1.05 \pm 0.03\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$, in good agreement with the value of $-1.09 \pm 0.08\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ reported by Thomson and Mehrishi[3]. However, in contrast to Thomson and Mehrishi[3] we find that the isoelectric point of the normal human erythrocyte occurs at pH 3.5 as opposed to pH 2.0 to 2.5. A possible explanation for the difference in isoelectric points may be the use of gelatin by Thomson and Mehrishi[3] to facilitate the sedimentation of red blood cells during their isolation procedure. As has been observed previously[28] there is some indication that the adsorption of polymeric materials to cell surfaces may lead to a change in their surface properties. The disagreement in the pH value for the isoelectric point may also be a reflection of differences in the surface properties of B and T lymphocytes. Our isolation procedure yields predominantly T lymphocytes and the various methods used by Mehrishi and Thomson[3] are likely to lead to varying mixtures of B and T lymphocytes which perhaps accounts also for the appreciable spread which they find in the isoelectric point. The ionogenic complexity of the lymphocyte surface precludes any easy estimate of the pK values of surface groups; however, the increase in anodic mobility at pH 10 and above indicates the presence of positive groups, probably amino groups.

The increase in mobility of the lymphocytes which occurs on fixation with formaldehyde (Fig. 2) shows that the surface negative charge is increased by about 10%. Blocking of the positive groups by aldehyde enables an approximate estimate of their pK to be made. The mobility of the untreated lymphocyte has increased to about half of the total increase which occurs on aldehyde fixation at pH 10, and it is therefore concluded that the pK of the positive groups occur at about pH 10. Examination of the low pH end of the pH *versus* mobility relationships for either formaldehyde or glutaraldehyde-fixed lymphocytes indicates a pK at about 3.2, a not unexpected result for a surface consisting of a mixture of carboxyl groups from sialic acid (pK 2.6) and acidic amino acid carboxyl groups (pK about 3.9)[19]. Treatment of fresh lymphocytes with glutaraldehyde leads, in addition to the same increase as observed for formaldehyde treatment, to the appearance of an additional

negative group with a pK of approximately 6.5. No satisfactory explanation for the appearance of the additional negative groups is forthcoming at present. A similar effect has been observed for human red blood cells after treatment with glutaraldehyde[25]. The pH *versus* mobility relationship for glutaraldehyde-fixed lymphocytes is identical below pH 6.0 with that for formaldehyde-fixed lymphocytes. There is no positive branch to the curves for formaldehyde- and glutaraldehyde-fixed lymphocytes at low pH values indicating the absence of aldehyde unreactive positive groups and of significant adsorption of hydrogen ions at low pH.

It is estimated that the pK of the groups remaining on the aldehyde-fixed neuraminidase-treated lymphocytes is about 4.3. It may be noted that less than half of the total negative charge of the cell is removed by the action of neuraminidase. Despite the scatter in the results, it appears, from the mean value at pH 7.0 $\pm$0.2 and the curve at lower pH values, that the increase in mobility which occurs above pH 6.5 for glutaraldehyde-fixed cells is still present after they have been treated with neuraminidase.

Both the charge densities in esu cm^{-2} and the number of electron charges per lymphocyte have been calculated and are listed in Table I. In order to calculate the actual number of electron charges per lymphocyte, an estimate of the surface area is necessary. Several estimates of lymphocyte volume are available in the literature and the mean value of four recent reports has been used in order to calculate the lymphocyte radius and hence surface area assuming a spherical shape. Volumes of 265 μm^3 (ref. 21), 250μm^3 (ref. 22), 265 μm^3 (ref. 23) and 269 μm^3 (ref. 24) have been reported for the human lymphocyte to yield a mean volume of 262 μm^3 and thus a surface area for the lymphocyte close to 200 μm^2. This figure is considerably larger than the surface area of 113 μm^2 used by Mehrishi[2]. Thus, although the charge densities obtained by Mehrishi[29] are in reasonable agreement with our estimates, the number of electron charges per cell are not.

In contrast to the above findings with fresh lymphocytes, Fig. 3 summarizes the results of investigation of surface properties of lymphocytes separated from stored blood. It is apparent that (a) the surface negative charge is significantly reduced between pH 6.0 and pH 9.5 and (b) there is a wide scatter in results from different blood samples and also a scatter within individual samples. Furthermore, there was a complete lack of pH reversibility with all stored lymphocytes tested.

The changes which we observe in human lymphocyte membranes during storage may be related to loss of function and an increase in chromosome and chromatid aberrations[30], or result from modification of the lymphocyte surface by proteolytic enzymes which arise from lysosomes released from degenerating polymorphonuclear leukocytes[31]. Other possibilities include the effects of cold storage[32] or the action of a stromal proteinase which has been proposed for the changes which also occur in the human erythrocyte membrane proteins during storage[33].

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada and in part by U.S. Public Health Service Grant HE-12787 from the National Heart and Lung Institute.

REFERENCES

1 Lundgren, G. and Simmons, R. L. (1971) *Clin. Exp. Immunol.* 9, 915–926
2 Mehrishi, J. N. (1972) *Int. Arch. Allerg.* 42, 69–77
3 Thomson, A. E. R. and Mehrishi, J. N. (1969) *Eur. J. Cancer* 5, 195-206
4 Lichtman, M. A. and Weed, R. I. (1970) *Blood* 35, 12–22
5 Seaman, G. V. F. and Heard, D. H. (1960) *J. Gen. Physiol.* 44, 251–268
6 Seaman, G. V. F. and Vassar, P. S. (1966) *Arch. Biochem. Biophys.* 117, 10–17
7 Vassar, P. S., Kendall, M. J. and Seaman, G. V. F. (1969) *Arch. Biochem. Biophys.* 135, 350–355
8 Seaman, G. V. F., Vassar, P. S. and Kendall, M. J. (1969) *Arch. Biochem. Biophys.* 135, 356–362
9 Vassar, P. S. and Culling, C. F. A. (1964) *Nature* 202, 610–611
10 Vassar, P. S., Taylor, H. E. and Culling, C. F. A. (1963) *Nature* 200, 691–692
11 Grothaus, E. A., Flye, M. W., Yunis, E. and Amos, D. B. (1971) *Science* 173, 542–544
12 Woodruff, J. J. and Gesner, B. M. (1969) *J. Exp. Med.* 129, 551–567
13 Ray, P. K., Gewurz, H. and Simmons, R. L. (1971) *Transplantation* 12, 327–329
14 Pease, D. C. (1964) *Histological Techniques for Electron Microscopy*, 2nd edn, p. 50, Academic Press, New York
15 Agostoni, A. and Idéo, G. (1965) *Experientia* 21, 82–83
16 Wilson, J. D. and Nossal, G. J. V. (1971) *Lancet* 2, 788–791
17 Greaves, M. F. and Hogg, N. M. (1971) in *Proc. Third Sigrid Jusélius Symp. on Cell Interactions and Receptor Antibodies in Immune Responses* (Makela, O., Cross, A. and Kosunen, T. U., eds), pp. 145–155, Academic Press, London and New York
18 Bangham, A. D., Flemans, R., Heard, D. H. and Seaman, G. V. F. (1958) *Nature* 182, 642–644
19 Haydon, D. A. and Seaman, G. V. F. (1967) *Arch. Biochem. Biophys.* 122, 126–136
20 Haydon, D. A. (1961) *Biochim. Biophys. Acta* 50, 450–457
21 Zucker, R. M. and Cassen, B. (1969) *Blood* 34, 591–600
22 Westring, D. W., Ladinsky J. L. and Feick, P. (1969) *Proc. Soc. Exp. Biol. Med.* 131, 1077–1083
23 Van Dilla, M. A., Fulwyler, M. J. and Boone, I. U. (1967) *Proc. Soc. Exp. Biol. Med.* 125 367–370
24 Lichtman, M. A. and Weed, R. I. (1969) *Blood* 34, 645–660
25 Vassar, P. S., Hards, J. M., Brooks, D. E., Hagenberger, B. and Seaman, G. V. F. (1972) *J. Cell Biol.* 53, 809–818
26 Zeiller, K., Holzberg, E., Pascher, G. and Hannig, K. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 105–110
27 Fotino, M., Merson, E. J. and Allen, F. H. (1971) *Vox Sang.* 21, 469–470
28 Brooks, D. E., Seaman, G. V. F. and Walter, H. (1971) *Nature, New Biol.* 234, 61–62
29 Mehrishi, J. N. (1970) *Eur. J. Cancer* 6, 127–137
30 Turner, J. H., Hutchinson, D. L. and Petricciani, J. (1971) *Scand. J. Haematol.* 8, 169–176
31 McCullough, J., Benzon, S. J., Yunis, E. J. and Quie, P. G. (1969) *Lancet* 2, 1333–1337
32 Collins, Z., Shepherd, L. P., Tarris, R. and Walford, R. L. (1970) *Transfusion* 10, 21–25
33 Moore, G. L., Cooper, D. A., Antonoff, R. S. and Robinson, S. L. (1971) *Vox Sang.* 20, 239–251