Biochimica et Biophysica Acta 902 (1987) 278–286
Elsevier

BBA 73641

Specific deposition of complement protein C3b on abnormal PNH erythrocytes permits their separation by partitioning. Possible general approach for isolation of specific cell populations

Michael K. Pangburn [a] and Harry Walter [b]

[a] *Department of Biochemistry, University of Texas Health Center, Tyler, TX 75710* and [b] *Laboratory of Chemical Biology, Veterans Administration Medical Center, Long Beach, CA 90822 and Department of Physiology and Biophysics, University of California, Irvine, CA 92717 (U.S.A.)*

(Received 5 February 1987)
(Revised manuscript received 15 May 1987)

Key words: Cell separation; Paroxysmal nocturnal hemoglobinuria; Aqueous phase system; Complement

The deposition of complement proteins on a cell surface has previously been shown to reduce the cell's partition ratio in a two-polymer aqueous phase system. This phenomenon has now been extended to segregate, by partitioning, subpopulations of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH). Purified components of the complement system were employed to deposit the protein C3b specifically on abnormal erythrocytes which lacked the membrane-associated complement regulatory protein DAF. As few as 2100 C3b / cell reduced the partition ratio and 24 000 C3b / cell resulted in resolution of the C3b-bearing and non-bearing human red cells. It was found that the proportion of cells separated did not equal the proportion of cells lysed by complement in the acidified serum lysis test when blood from three of the five patients was examined. The results indicate that the defect giving rise to DAF$^-$ cells may be, but is not necessarily, coexpressed with defects affecting other membrane-associated regulatory factors. A broader application of the method using monoclonal antibodies to direct purified complement components to specific cell populations should permit their isolation in large quantities.

Introduction

Aqueous solutions of dextran and of poly(ethylene glycol) when mixed above certain concentrations give rise to immiscible, liquid two-phase systems with a poly(ethylene glycol)-rich top and a dextran-rich bottom phase [1]. Such systems, being aqueous, can be buffered and made isotonic and are suitable for the separation of viable cells by partitioning [2,3]. The partition ratio of cells (defined as the quantity of cells in the top phase as a percentage of total cells added) [4] depends sensitively on the cell's surface properties [2].

We have previously found that the partition ratio of sheep erythrocytes is unaffected by coating them with subagglutinating quantities of anti-sheep red blood cell antibody while the further addition of sub-lytic quantities of complement results in lower cell partition ratios [5]. These results led us to test the possibility of obtaining, by partitioning, specific cell populations or subpopulations by means of the directed, specific

Abbreviations: PNH, paroxysmal nocturnal hemoglobinuria; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; DAF, decay accelerating factor.

Correspondence: M.K. Pangburn, Department of Biochemistry, University of Texas Health Center, Tyler, TX 75710, U.S.A.

deposition of complement protein C3b on the cell surface of interest.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia which is thought to arise due to a somatic mutation. The formation of clones of abnormal stem cells in the bone marrow gives rise to variable amounts of abnormal erythrocytes that are unusually sensitive to the hemolytic action of complement [6,7]. Abnormal granulocytes and platelets are also produced [8,9].

During complement activation PNH erythrocytes bind more C3b [10,11] and mroe C5 [12] than do normal erythrocytes. These observations are consistent with the finding that abnormal PNH erythrocytes lack the membrane-associated protein DAF [11,13,14]. DAF (decay accelerating factor) is a 73 kDa single-chain glycoprotein which has been isolated from normal human erythrocytes [15]. Its function is to prevent formation of and to inactivate C3 and C5 convertases of both pathways of complement [16,17], thereby preventing lysis of the cells. Inhibition of DAF activity on normal erythrocytes with monospecific antibodies induces increased sensitivity to complement [11] and introduction of purified DAF into PNH erythrocytes reduces their sensitivity to lysis by complement [18]. In the most severe form of PNH, the abnormal erythrocytes may lack an additional complement regulatory factor or factors present in normal erythrocytes [19–25]. In the present report large numbers of C3b molecules were attached to cells lacking the regulatory activity of DAF. The presence of high numbers of surface-bound C3b resulted in an appreciable diminution of the cells' partition ratio allowing separation of the abnormal cells from DAF^+ erythrocytes by countercurrent distribution.

Materials and Methods

Purified proteins and reagents. C3 [26], Factors B [27], D [28], and nephritic factor [29] were prepared as previously described. TPCK-trypsin was purchased from Worthington Biochemical Corp. Freehold, N.J. Radioiodination of C3 and Factor H was performed according to the method of Fraker and Speck [30] with Iodogen (Pierce Chem. Co., Rockford, IL). MgEGTA contained 0.1 M $MgCl_2$ and 0.1 M EGTA at pH 7.2. Dextran T500, lot no. GI 21917, was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, and poly(ethylene glycol) 8000 (trade name 'Carbowax 8000') from Union Carbide, New York. ^{51}Cr-chromate was the product of ICN Irvine, CA and fetal bovine serum was obtained from GIBCO, Grand Island, NY.

Lysis of PNH erythrocytes in acidified serum. The acidified serum test for PNH [31] was performed as described previously [11]. Normal human serum was made 10 mM in Bistris (bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane, Sigma Chemical Co., St. Louis, MO) by addition of 1% v/v of a 1 M stock solution of this buffer at pH 6.0. The serum was then adjusted to pH 6.4 with 1 M HCl immediately prior to use. Erythrocytes were washed three times with pH 6.4 GVB and 10 μl (approx. 10^7 cells) mixed with 60 μl pH 6.4 serum and 30 μl of 5 mM veronal, 150 mM NaCl, 8.3 mM $MgCl_2$ and 8.3 mM EGTA at pH 6.4. After 30 min at 37°C, lysis was stopped by the addition of 0.9 ml cold 5 mM veronal, 150 mM NaCl, 10 mM EDTA and 0.1% gelatin at pH 7.4. Unlysed cells were removed by centrifugation and lysis was assessed by measuring the absorbance of released hemoglobin at 412 nm. Assays containing 20 mM EDTA or 0.1% Nonidet 40 (Sigma Chemical Co.) were used to determine minimum or maximum lysis, respectively. Complete lysis of sheep erythrocytes and the absence of lysis of normal human erythrocytes [13] were necessary controls since sera vary in their effectiveness in this assay.

Determination of erythrocyte concentrations. The concentration of erythrocytes was determined following separation by countercurrent distribution by measuring the absorbance of hemoglobin at 540 nm after lysis of the cells.

Deposition of C3b on human erythrocytes. C3b deposition was performed as previously described [11,13]. Washed human erythrocytes with a packed volume of 0.5 ml (approx. $5 \cdot 10^9$) were resuspended in 150 μl of C3 containing 1.8 mg C3. Trypsin (5 μl of 1 mg/ml trypsin) was added to activate C3 producing metastable C3b a small proportion of which attaches randomly to cells [17]. After a 6 min incubation at 22°C the cells were washed five times in 5 mM veronal, 150 mM NaCl containing 0.1% gelatin (pH 7.4). This pro-

cedure deposits between 100 and 500 C3b/cell. A mixture (50 μl) containing 18 μg Factor B, 0.5 μg Factor D and 25 mM $NiCl_2$ was added to the pellet of cells, mixed and allowed to incubate 3 min at 22° C. This allows formation of cell-bound C3 convertase. Enzyme formation is considerably more efficient on the abnormal cells. Formation is stopped by addition of 20 μl of 0.2 M EDTA (pH 7.4) and the C3 convertase is allowed to decay for 4 min at 22° C during which most of the enzymes formed on normal cells are inactivated by DAF, but PNH erythrocytes lacking DAF retain more than 80% of the surface-bound enzyme [11]. The surviving enzymes were stabilized by addition of an excess of nephritic factor (100 μg in 5 μl). Addition of 0.3 to 2.3 mg C3 containing 5 μCi ^{125}I-labeled C3 results in deposition of large numbers of C3b molecules onto the cells bearing active enzyme. After 2 h incubation at 37° C the cells were washed four times in 5 mM veronal, 150 mM NaCl and 0.1% gelatin (pH 7.4) and resuspended at $1 \cdot 10^9$/ml.

In order to deposit C3b on normal human erythrocytes the above procedure was modified by premixing nephritic factor with the 50 μl of Factor B, Factor D and $NiCl_2$ and by eliminating the 4-min decay following EDTA addition. These modifications essentially prevent the DAF-mediated inhibition of enzyme formation and of enzyme decay, thereby permitting C3b deposition on normal human erythrocytes equivalent to that obtained with abnormal PNH erythrocytes.

Determination of the number of C3b per cell. The average number of C3b molecules bound per human erythrocyte was determined as previously described [32]. This method utilizes binding of radiolabeled Factor H to cell-bound C3b under standardized conditions to measure the amount of C3b present. It takes into account the effect of clustered C3b on the binding of Factor H, but may underestimate the number of C3b on PNH erythrocytes [33].

^{51}Cr-labeling of erythrocytes. Normal red blood cells or those with different quantities of C3b on their surface, were labeled with [^{51}Cr]chromate as previously described [34]. Ten to 15 μCi of ^{51}Cr was used to label 0.2 ml of red blood cells suspended in 2 ml of saline. Cells were washed five times with phosphate-buffered saline after the labeling procedure to remove unbound isotope.

Preparation of mixtures of ^{51}Cr-labeled and unlabeled cells for countercurrent distribution. ^{51}Cr-labeled red cells, with or without C3b on their surface, were mixed in a ratio of 1:4 with unlabeled, thrice washed human normal red blood cells with which their partition ratios were to be compared in a manner analogous to that previously used for the comparison of other erythrocyte populations [34–36]. The effect of ^{51}Cr-labeling on the partition of C3b-bearing erythrocytes was examined by mixing erythrocytes bearing C3b with a small number of identical, but ^{51}Cr-labeled erythrocytes.

Preparation and composition of phase systems. Two phase systems, selected and prepared as previously described [2] were used. Phase system 1 was employed in the initial experiments and contained 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol), 0.105 M sodium phosphate buffer (composed of equimolar concentrations of mono and dibasic sodium phosphates), pH 6.8, and 5% (w/w) heat-inactivated fetal bovine serum. Phase system 2 had the composition 5% (w/w) dextran, 3.8% poly(ethylene glycol) and 0.11 M sodium phosphate buffer (pH 6.8). Phase system 2 was used to assure ourselves that the presence of heat-inactivated fetal bovine serum in system 1 did not influence the results. Phase systems were permitted to equilibrate in a separatory funnel at 4–5° C. Top and bottom phases were then separated.

Both systems have electrostatic potential differences between the top and bottom phases and are thus charge-sensitive (see Ref. 2 for detailed discussion of the physical properties of the phases and the surface properties of cells that determine the cell partition ratio).

Countercurrent distribution of cells and cell mixtures. Our automatic countercurrent distribution apparatus (Workshop, Chemical Center, University of Lund, Sweden) has circular Plexiglas plates with 120 concentric cavities and a bottom phase capacity of 0.7 ml [37]. All cavities received 0.5 ml of bottom phase of the system to be used in a given separation. 0.2 ml of the washed, packed red cells or red cell mixtures (prepared as described above) were suspended in 2.2 ml of corresponding top phase ('load mix'). When three different sam-

ples were to be run separately but simultaneously, cavities 0–2, 40–42 and 80–82 each received 0.7 ml of one of the load mixes while cavities 3–39, 43–79 and 83–119 received 0.7 ml of top phase. When four different samples were to be run, the cavities receiving load mixes were 0–2, 30–32, 60–62, and 90–92 with all others receiving top phase. Countercurrent distribution was then performed on the automatic unit at 4–5 °C as previously described [34] using a 6 min settling time and a 25 s shaking time. Forty transfers were completed when three samples were run and 30 transfers with four samples.

Analysis of cells after countercurrent distribution. After separation cells were collected directly into plastic centrifuge tubes. Selected tubes (usually every other) were analyzed. An isotonic aqueous salt solution (0.7 ml) was added to each tube and they were centrifuged. The supernatant solution was discarded and 1 ml of 20 mosM sodium phosphate buffer (pH 7.2) was added to lyse the cells. In experiments without isotopic label or with ^{51}Cr-labeled cells, the lysate was centrifuged at high speed to remove the storma and the hemoglobin absorbance of the supernatant solution was measured at 540 nm. In experiments containing ^{125}I-C3b the isotope was counted prior to high speed centrifugation and removal of storma.

Presentation of data. Cell distributions are given in terms of hemoglobin absorbance at 540 nm [34] and distributions of isotopically labeled cells in counts per minute (cpm). In experiments in which mixtures of ^{51}Cr-labeled and unlabeled cells of the same type are examined by countercurrent distribution, the total cell distribution (in hemoglobin absorbance) indicates predominantly the unlabeled cell population since these are the cells present in great excess while the labeled cell population is, again, in cpm [34].

Results

Human erythrocytes bearing surface-bound C3b have reduced partition ratios

Labeling human erythrocytes with [^{51}Cr]chromate does not affect their partition ratio [34–36]. Furthermore, the ^{51}Cr-labeled cells accurately reflect the distribution profile of both unmodified erythrocytes and erythrocytes bearing C3b as illustrated by the coincident curves for total hemoglobin and ^{51}Cr label in Figs. 1A and 1B.

Fig. 2 illustrates the sensitivity of the partition

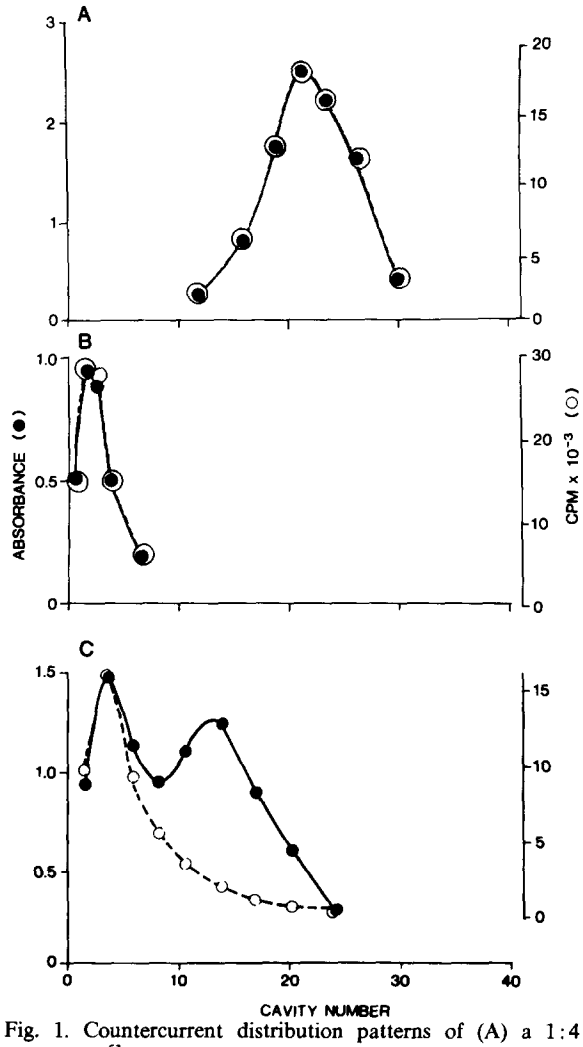

Fig. 1. Countercurrent distribution patterns of (A) a 1:4 mixture of ^{51}Cr-labeled and unlabeled normal human erythrocytes; (B) a 1:4 mixture of ^{51}Cr-labeled and unlabeled normal human erythrocytes both bearing 58000 C3b/cell (deposited as described in the text); and (C) a 1:4 mixture of ^{51}Cr-labeled cells as in (B) and unlabeled normal human erythrocytes. The three preparations were subjected to countercurrent distribution, separately but simultaneously, in a charge-sensitive dextran-poly(ethylene glycol) aqueous phase system. Phase system composition: 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol), 0.105 M sodium phosphate buffer (pH 6.8) and 5% (w/w) heat-inactivated fetal bovine serum. Forty transfers were completed at 4–5 °C using a 6 min settling and a 25 s shaking time. ●, total cell distribution (in terms of hemoglobin absorbance at 540 nm); ○, distribution of labeled cells (in cpm). For additional details and discussion see text.

ratio to the presence of C3b. Fig. 2A depicts the distribution curve of normal human erythrocytes in the indicated phase system. Fig. 2B shows that erythrocytes which have been coated with only 2100 molecules per cell of the complement protein C3b (and labeled with ^{51}Cr) have a lower partition ratio than cells from the same normal individual without C3b. The extent to which the partition ratio is reduced was found to depend on the number of C3b molecules bound per cell (Fig. 2C). The experiments shown in Fig. 1 demonstrate that normal erythrocytes (Fig. 1A) and the same cells bearing 58000 surface-bound C3b per cell (Fig. 1B) have dramatically different partition ratios.

Fig. 1C demonstrates that upon mixing un-

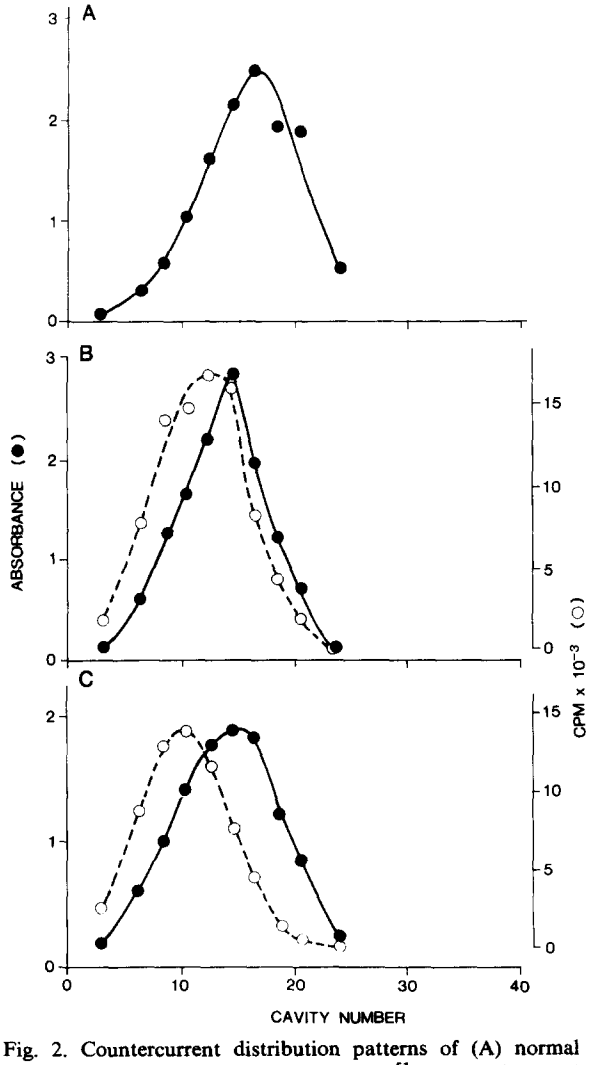

Fig. 2. Countercurrent distribution patterns of (A) normal human erythrocytes; (B) a 1:4 mixture of ^{51}Cr-labeled normal human erythrocytes on which 2100 C3b/cell had been deposited and normal human erythrocytes; and (C) a mixture as in (B) except that 5300 C3b had been deposited on the labeled cells. Other conditions and symbols as in Fig. 1.

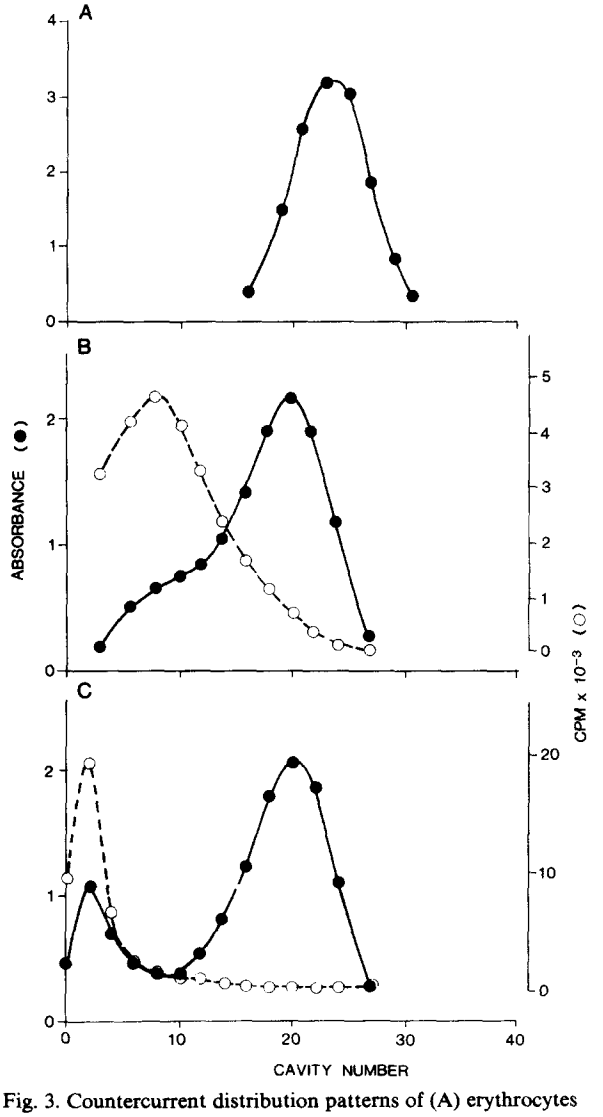

Fig. 3. Countercurrent distribution patterns of (A) erythrocytes from PNH patient DM; (B) erythrocytes as in (A) except that 24000 ^{125}I-labeled C3b molecules had been specifically deposited on the abnormal red cells (as described in the text); and (C) experiment as in (B) except that 43000 ^{125}I-labeled C3b molecules had been deposited on the abnormal cells. Phase system composition: 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol) and 0.11 M sodium phosphate buffer (pH 6.8). Other conditions and symbols as in Fig. 1.

labeled normal erythrocytes with cells bearing C3b the cells retain their distinct partition ratios but exhibit reproducible shifts in the ratios of both populations suggesting that these cell populations interact. This is not unexpected since human erythrocytes express on their surface the C3b receptor CR1, also known as the immune adherence receptor [38]. Cell–cell interaction have previously been shown to be detectable by countercurrent distribution [39].

Abnormal PNH erythrocytes selectively permit C3b deposition and exhibit lower partition ratios

The proportion of abnormal cells in PNH blood which lack the complement regulatory protein DAF have been shown [11] to allow unregulated attachment of C3b. The cells bearing normal amounts of DAF inactivate the depositing enzyme and when treated as described in Methods receive little C3b. Figs. 3 and 4 show the results of treating erythrocytes from two PNH patients with complement and subjecting the cells to countercurrent distribution. The untreated cells from patient DM (Fig. 3A) partitioned as a single species while the complement-treated cells split into two populations (Fig. 3B). The radiolabel migrated exclusively with the population with lower partition ratio indicating that these cells bore the C3b (24000 C3b/cell). By increasing the amount of C3 offered to the cells more C3b could be deposited and two distinct populations were resolved (Fig.

TABLE I

COMPARISON OF THE PERCENTAGE OF ERYTHROCYTES SHIFTED ON COUNTERCURRENT DISTRIBUTION WITH THE PERCENTAGE LYSED BY COMPLEMENT

Individual	% Lysis	% Shifted
PNH patient		
CR	14	18
DM	13	20
DS	40	83
EH	30	92
VR	54	26
Normal		
MP	0	0
HW	0	0

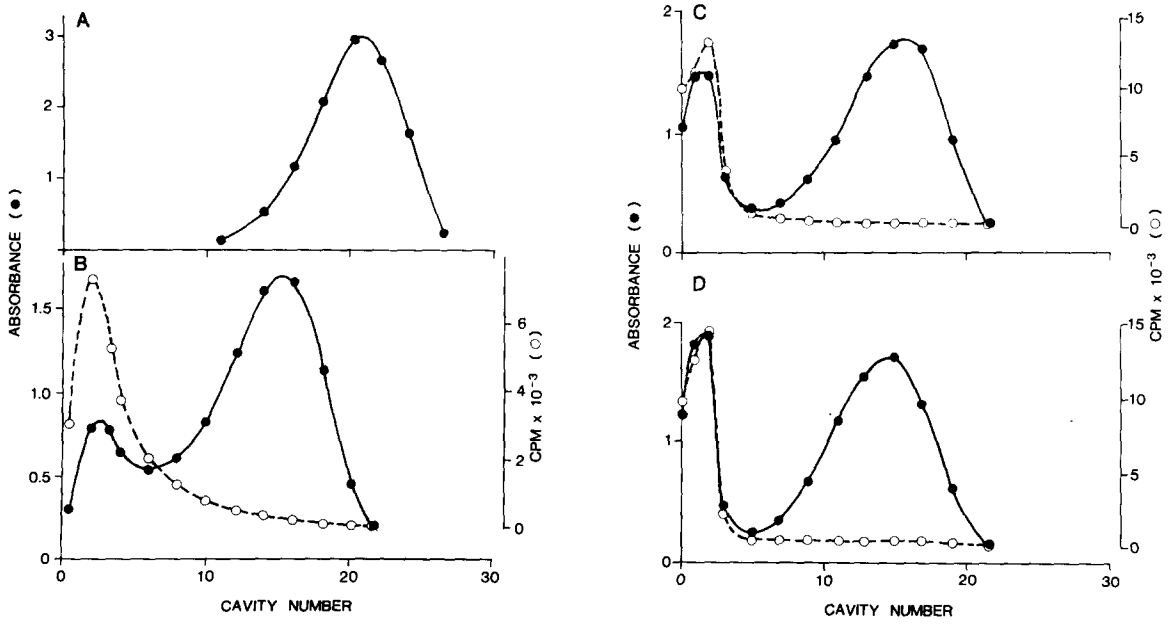

Fig. 4. Countercurrent distribution patterns of (A) erythrocytes from PNH patient VR; (B) erythrocytes as in (A) except that 22000 ^{125}I-labeled C3b molecules had been specifically deposited on the abnormal red cells; (C) experiment as in (B) except that 39000 ^{125}I-labeled C3b molecules had been deposited on the abnormal cells; and (D) experiment as in (B) except that 67000 ^{125}I-labeled C3b molecules had been deposited on the abnormal cells. Thirty transfers were completed. Other conditions and symbols as in Fig. 3.

3C, 43000 C3b/cell). Approximately 20% of the total erythrocytes were found in the population with lower partition ratio which corresponds well with the proportion of cells that lysed in acidified serum, a clinical test for PNH. Table I indicates that with three of the five patients these values did not agree, suggesting that the expression of DAF and the expression of other factors which regulate lysis by homologous complement [19–25] are not unalterably linked. As seen in Figs. 1 and 2 there was a small but reproducible shift in the unlabeled population, probably due to an interaction of the C3b receptor with C3b. This is also evident in Fig. 4 which shows the partitioning profiles of cells from patient VR. In panels B, C and D the shifted cells bore 22000, 39000 and 67000 C3b/cell, respectively. Similar treatment with complement of erythrocytes from normal individuals resulted in the acquisition of fewer than 250 C3b/cell. The countercurrent distribution pattern of these cells showed neither a shift (Table I) nor a bimodal distribution.

Discussion

Unlike most other separatory methods the parameters that affect cell separation are reflected in a most sensitive manner in countercurrent distribution because their relation to the partition ratio is exponential [2]. For example, surface charge can be a determinant in the partitioning behavior of cells in aqueous phase systems and charge is thus exponentially related to the partition ratio while in electrophoresis the relation of charge to cell mobility is linear. Furthermore, the number of cells that can be processed on our apparatus is much larger than in fluorescence-activated cell sorting or free-flow electrophoresis [40]: on the order of 10^{10} cells of the size of erythrocytes (or 10^9 lymphoctyes). If the partition ratios of cells to be segregated are adequately different the desired cells are obtainable in a few batch extraction steps, permitting the process to be scaled up even further.

The extraction of specific cells by partitioning has recently been accomplished in model experiments by utilizing antibody-poly(ethylene glycol) conjugates [41,42] which partition into the poly(ethylene glycol)-rich phase. Specific erythrocytes to which the antibody was directed were extracted from an artificial mixture of red blood cells. The limitations of this procedure are that the method is only applicable in cases in which an adequately large number of antibody binding sites with adequate binding constants is present and the need to synthesize a specific poly(ethylene glycol)-antibody for every cell type one wishes to extract.

The approach used in the present study takes advantage of the ability of complement to amplify a small initial signal and to deposit a large number of protein molecules on the target cell. By employing specific purified components of the complement system the cytolytic effects of complement are avoided and the system may be manipulated to deposit any number of C3b molecules on the cells. Fig. 2 shows that the partition ratio of human erythrocytes is reduced by the presence of as few as 2100 C3b molecules per cell and Fig. 3 demonstrates that cells bearing 24000 C3b are separated from cells lacking C3b.

C3b was selectively deposited in this study onto abnormal PNH erythrocytes lacking the regulatory activity of DAF. Erythrocytes from normal individuals and the normal erythrocytes in PNH blood do not acquire significant numbers of C3b during these procedures due to inhibition by DAF of the C3b-depositing enzyme. Five PNH patients were examined and all possessed abnormal erythrocytes which acquired large numbers of C3b. Countercurrent distribution effectively separated cells with C3b on their surface from those without C3b as indicated (Figs. 3C, 4C and 4D) by the association of virtually all the radioactivity (^{125}I-C3b) with cells having the lower partition ratio.

All four Figs. provide evidence that receptor-ligand interactions have occurred during countercurrent distribution. The interaction of the C3b receptor on normal erythrocytes with C3b bound to the abnormal cells is most probably the cause of the shift in partition ratios of the two populations. It has previously been shown that countercurrnet distribution can be a useful tool in discovering or tracing cell-cell affinities [39].

The physical separation of PNH erythrocytes expressing DAF activity from cells lacking this activity shows that the proportion of C3b-coated cells is not always quantitatively the same as the proportion of cells lysed by complement (Table I).

Three specific deficiences have, thus far, been demonstrated in PNH erythrocytes: acetylcholinesterase [43], DAF [11,13,14] and a recently identified C5-9 inhibitor [19–25]. Our results and the results of others [44–46] suggest that expression of any one of these proteins is not firmly linked to expression of the others. Two recent reports support this conclusion [45,46]. Monoclonal antibodies to acetylcholinesterase were used to separate esterase-negative from esterase-positive cells. Medof et al. [45] reported the identification of acetylcholinesterase-postive erythrocytes (PNH I) which were partially DAF deficient. Chow et al. [46] reported two types of PNH III erythrocytes, one of which exhibited properties which might be expected for cells bearing DAF, but lacking the C5-9 inhibitor. PNH II erythrocytes which lack acetylcholinesterase and DAF are often present in the blood of patients containing both PNH I and PNH III erythrocytes, the latter most commonly lacking all three membrane proteins. Although the molecular mechanism of this diversity is unclear, these observations necessitate consideration of more patterns of expression or the participation of more factors than previously thought.

PNH provides a unique type of cell which lacks a membrane-associated complement regulatory factor. Clearly, this method of separating cells would be of limited usefulness if specific deposition of C3b were limited to DAF-deficient cells. The real potential of the finding that C3b markedly reduces cell partition ratios is that C3b deposition may be directed at particular cells by use of monoclonal antibodies. A 100-fold amplification is easily achieved, suggesting that as few as 500 antigenic sites on a cell would be sufficient to alter the partition ratio, although larger cells may require more initial sites or greater amplification. The number of C3b per cell is not limiting since more than $2 \cdot 10^6$ C3b may be deposited on, for example, erythrocytes [47]. The presence of C3b does not have a deleterious effect on nucleated or non-nucleated cells in the absence of other complement components. C3b may be removed after countercurrent distribution by cleavage of the ester linkage [48,49], by degradation by C3b-specific regulatory proteins of complement [50] or, in the case of nucleated cells, by normal membrane turnover during culturing.

Acknowledgments

We thank Eugene J. Krob, Kerry L. Wadey-Pangburn and Reginald L. Tyiska for their expert technical assistance. We also thank Doctors R.H. Bigley, M.E. Dockter, P. Kenyon, M., Morrison, G.V.F. Seaman and D. Stoltzman for their assistance in obtaining PNH blood samples for this study. This work was supported by United States Public Health Service Grant DK-35081 and by the Medical research Service of the Veterans Administration. The work was done during the tenure of an Established Investigatorship to Michael K. Pangburn from the American Heart Association and with funds contributed in part by the American Heart Association, Texas Affiliate.

References

1 Albertsson, P.-Å. (1986) Partition of Cell Particles and Macromolecules. 3rd Edn., Wiley-Interscience, New York
2 Walter, H. (1985) in Partitioning in Aqueous Two-phase Systems. Theory, Methods, Uses, and Applications to Biotechnology (Walter, H., Brooks, D.E. and Fisher, D., eds.), pp. 327–376, Academic Press, Orlando, FL U.S.A.
3 Walter, H. and Johansson, G. (1986) Anal. Biochem. 155, 215–242
4 Albertsson, P.-Å, and Baird, G.D. (1962) Exp. Cell Res. 28, 296–322
5 Walter, H. and Krob, E.J. (1979) Cell Biophys. 1, 149–160
6 Rosse, W.F. (1978) Clin. Haematol. 7, 541–553
7 Rosse, W.F. (1972) in Hematology (Williams, W.J., Beutler, E. Ersley, A.J. and Rundles, R.W., eds.), pp. 460–474, McGraw-Hill, New York
8 Dixon, R.H. and Rosse, W.F. (1977) J. Clin. Invest. 59, 360–368
9 Aster, R.V. and Enright, S.E. (1969) J. Clin. Invest. 48, 1199–1210
10 Logue, G.L., Rosse, W.F. and Adams, J.P. (1973) J. Clin. Invest. 52, 1129–1137
11 Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5430–5434
12 Götze, O. and Müller-Eberhard, H.J. (1970) J. Exp. Med. 132, 898–915
13 Pangburn, M.K., Schreiber, R.D., Trombold, J.S. and Müller-Eberhard, H.J. (1983) J. Exp. Med. 157, 1971–1980
14 Nicholson-Weller, A., March, J.P., Rosenfeld, S.I. and Austen, K.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5066–5070
15 Nicholson-Weller, A., Burge, J., Fearon, D.T., Weller, P.F. and Austen, K.F. (1982) J. Immunol. 129, 184–189
16 Reid, K.B.M. and Porter, R.R. (1981) Annu. Rev. Biochem. 50, 433–464
17 Pangburn, M.K. and Müller-Eberhard, H.J. (1984) Springer Semin. Immunopathol. 7, 163–192

18 Medof, M.E., Kinoshita, T., Silber, R. and Nussenzweig, V. (1985) Proc. Natl. Acad. Sci. USA 82, 2980–2984
19 Zalman, L.S., Wood, L.M. and Müller-Eberhard, H.J. (1985) Proc. Natl. Acad. Sci. USA 83, 6975–6979
20 Shin, M., Hänsch, G., Hu, V.W. and Nicholson-Weller, A. (1986) J. Immunol. 136, 1777–1782
21 Rosenfeld, S.I., Jenkins, D.E. and Leddy, J.P. (1986) J. Exp. Med. 164, 981–989
22 Schönermark, S., Rauterberg, E.W., Shin, M.L., Löke, S., Roelcke, D. and Hänsch, G.M. (1986) J. Immunol. 136, 1772–1782
23 Jenkins, D.E. and Leddy, J.P. (1985) J. Immunol. 134, 506–511
24 Hu, V.W. and Nicholson-Weller, A. (1985) Proc. Natl. Acad. Sci. USA 82, 5520–5524
25 Schönermark, S., Roelcke, D. and Hänsch, G.M. (1986) Progress in Immunology 6, 191 (Abstr.)
26 Hammer, C.H., Wirtz, G.H., Renfer, L., Greshma, H.D. and Tack, B.F. (1981) J. Biol. Chem. 256, 3995–4006
27 Götze, O. and Müller-Eberhard, H.J. (1971) J. Exp. Med. 134, 90s–108s
28 Lesavre, P.H., Hugli, T.E., Esser, A.F. and Müller-Eberhard, H.J. (1979) J. Immunol. 123, 529–534
29 Schreiber, R.D., Götze, O. and Müller-Eberhard, H.J. (1975) J. Exp. Med. 142, 760–772
30 Fraker, P.J. and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. 80, 849–855
31 Dacie, J.V. and Richardson, N. (1943) J. Pathol. Bacteriol. 55, 375–378
32 Pangburn, M.K. and Müller-Eberhard, H.J. (1978) Proc. Natl. Acad. Sci. USA 75, 2416–2420
33 Parker, C.J., Baker, P.J. and Rosse, W.F. (1983) J. Immunol. 131, 2484–2489
34 Walter, H. and Krob, E.J. (1983) Cell Biophys. 5, 205–219 and 301–306
35 Walter, H. and Krob, E.J. (1984) Biochem. Biophys. Res. Commun. 120, 250–255
36 Walter, H., Levin, E.R., Krob, E.J. and Mills, S.D. (1986) Hypertension 8, 533–539
37 Albertsson, P.-Å. (1970) Sci. Tools 17, 53–57
38 Fearon, D.T. (1983) Springer Semin. Immunopathol. 6, 159–172
39 Walter, H., Krob, E.J. and Moncla, B.J. (1978) Exp. Cell Res. 115, 379–385
40 Hansen, E. and Hannig, K. (1982) J. Immunol. Methods 51, 197–208
41 Karr, L.J., Shafer, S.G., Harris, M.J., Van Alstine, J.M. and Snyder, R.S. (1986) J. Chromatogr. 354, 269–282
42 Sharp, K.A., Yalpani, M., Howard, S.J. and Brooks, D.E. (1986) Anal. Biochem. 154, 110–117
43 DeSandre, G. and Ghiotto, G. (1960) Br. J. Haematol. 6, 39–46
44 Rosse, W.F. and Parker, C.J. (1985) Clin. Hematol. 14, 105–125
45 Medof, M.E., Nussenzweig, V., Kinoshita, T., Hall, S., Gottlieb, A., Silber, R. and Rosse, W.F. (1985) Complement 2, 53 (Abstr.)
46 Chow, F.-L., Hall, S.E., Rosse, W.F. and Telen, M.J. (1986) Blood 67, 893–897
47 Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1983) J. Immunol. 131, 1930–1935
48 Pangburn, M.K. and Müller-Eberhard, H.J. (1980) J. Exp. Med. 152, 1102–1114
49 Janatova, J., Tack, B.F. and Prahl, J.W. (1980) Biochemistry 19, 4479–4485
50 Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1977) J. Exp. Med. 146, 257–270