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IMMUNO-AFFINITY PARTITION OF CELLS IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

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SUMMARY

Poly(ethylene glycol) (PEG) was covalently coupled to IgG antibody preparations directed against human red blood cells. This modification reduces the tendency of the antibody to agglutinate cells and increases its affinity for the upper phase in dextran–PEG aqueous two-phase systems. These effects are related to the molecular weight of the PEG used for modification and to the number of PEG molecules attached to the antibody.

Exposure of human red blood cells to PEG-modified antibody causes a substantial and specific increase in cell partition into the PEG-rich phase in a number of PEG–dextran aqueous two-phase systems. Pertinent phase-system parameters were examined. Following a single incubation with PEG-derivatized antibody, a mixture of sheep and human red blood cells was completely separated in 100 min by a 30-transfer countercurrent extraction using a two phase system which normally offers little resolution.

INTRODUCTION

There is considerable need both in biomedical research and in diagnostic medicine for reliable techniques for specifically separating a discrete population of cells from a mixture. The ideal technique should provide a good yield of cells of high purity while cell function is maintained. The present work indicates that immuno-affinity phase partition has the potential for becoming such a technique.

Aqueous solutions of low concentrations of the polymers dextran and poly(ethylene glycol) (PEG) form two-phase systems consisting of a less dense, PEG-rich phase floating on a dextran-rich phase. When buffered and made isotonic by the addition of salts or other low molecular weight solutes, these aqueous polymer two-phase systems can be used for analytical and preparative separation of a wide range of biological substances including cells, organelles, nucleic acids, and proteins[1–4]. Separation results from differential partitioning of substances between the two phases

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or, in the case of particles, between the liquid–liquid interface and either of the phases. Resolution can be enhanced by the repeated partitioning provided by countercurrent distribution and countercurrent chromatography[1–7].

Factors which influence the partition of macromolecules and particles include size, surface charge, and surface hydrophobicity[1–3]. System characteristics such as interfacial tension and salt composition also influence partition and can be altered by changing the type and concentration of polymers and salts[1–3].

Another method for altering partition is through the use of affinity ligands[7–15]. A ligand that selectively binds to a particle or macromolecule of interest is covalently coupled to one of the polymers (typically PEG). When mixed in a two-phase system, and allowed to settle, the PEG-bound ligand partitions predominately into the phase rich in that polymer, pulling the target material with it[8–15]. These ligands may be specific, such as enzyme inhibitors for enzyme purification[8], or nonspecific, like dyes for enzyme or nucleic acid purification[9–12] or fatty acid esters for cell purification[15].

The present article describes how we have refined the technique of phase partitioning to include specific binding of cell surface antigens by antibody ligands attached to PEG. Several steps are required to demonstrate the efficacy of immuno-affinity cell partition: (1) PEG must be attached to the antibody; (2) PEG-derivatized antibody (PEG-Ab) must partition into the PEG-rich phase and it must remain active; (3) the PEG-Ab must pull the cells of interest into the top phase; and (4) this cell partitioning must be selective for the targeted cells.

We have chosen to examine the partitioning of human and sheep red blood cells (RBCs) as a simple model system. RBCs have been extensively studied in phase partitioning[1–3] and have the additional advantage of being easily quantified; in addition, native antibodies are commercially available for immuno-affinity partitioning of these RBCs. Also, one aspect of antibody activity can easily be measured by microtiter hemagglutination assay; but it should be noted that PEG-modified antibody may be rendered effectively monovalent, and thus may still be active and capable of binding RBCs when it can no longer agglutinate RBCs. We chose a polymer phase system in which both human and sheep RBCs partitioned in favor of the interface and then used PEG-bound antibodies to the cell membrane of the human RBC to specifically increase the partition of human RBCs into the top, PEG-rich phase. The effects of phase-system parameters and the influence of PEG molecular weight and degree of derivatization were also investigated. In addition, we have examined the usefulness of PEG-antibodies in countercurrent distribution of cells.

MATERIALS AND METHODS

Unless specified otherwise all reagents were ACS grade, or better, quality from commercial sources. Distilled, filtered, 12 MΩ/cm water was used throughout.

Antibody preparations

Rabbit IgG directed against stroma from human RBCs (native antibody) was obtained from Cooper Biomedical, Malvern, PA, U.S.A. (lot 22196). Fab fragments derived from this same lot were prepared by controlled papain digestion by Jackson Immunoresearch, Avondale, PA, U.S.A. An enriched IgG fraction from sheep, also directed against human RBCs, was kindly provided by Dr. D. E. Brooks, University

of British Columbia, Vancouver, Canada. This fraction, which had been derived from whole serum by ammonium sulfate precipitation, was then further purified by ion exchange chromatography using a DEAE-silica gel column[16]. Both IgG samples agglutinated human RBCs at concentrations less than 0.2 μg/ml but did not agglutinate sheep RBCs.

Modification of antibodies with PEG

In a typical preparation, 12 mg of protein in 0.5 ml 0.05 *M* $Na_2B_4O_7$, pH 9.2, was added to 1.5 ml 0.1 *M* borate buffer. A volume of 1 ml of cyanuric chloride-activated[17,18] PEG 5000 or PEG 1900 monomethyl ether in the borate buffer was then added at 4°C, and the mixture was stirred for 1 h. Unattached PEG was removed by diafiltration (50-ml Amicon cell, PM-30 membrane, 30 000 molecular weight cut-off) with 10 volumes of 0.05 *M* borate buffer and 0.025 *M* sodium azide to a final volume of 2 ml. Typically 27 mg of activated PEG 5000, approximately equimolar relative to IgG lysine amino groups (90 per molecule)[19] resulted in about 50% modification of the lysine amino groups; only 70 of the 90 lysine groups were sensitive to our method of analysis (below). About half this amount of activated PEG (14 mg) gave 30% modification.

Analysis was performed by the Biuret and Habeeb methods as described previously[17,18]. For the latter method (based on reaction of trinitrobenzenesulfonic acid with free lysine groups), we found it necessary to remove unattached PEG and to keep protein concentrations at approximately 0.6 mg/ml in order to obtain accurate and reproducible results. The 0.1 *M* borate buffer was used rather than 4% $NaHCO_3$ called for in the original procedure.

RBC preparation

Blood samples were obtained by venipuncture from healthy individuals and treated with 10 m*M* EDTA as an anticoagulant. Samples were used fresh following brief storage at 4°C. Shortly before use, 1-ml aliquots were washed four times by suspension in twenty volumes of isotonic buffer (Isoton II, Coulter Electronics, Hialeah, FL, U.S.A.) and centrifugation for 10 min at 1000 *g*, followed by removal of the supernatant and the buffy coat.

RBC quantification

Quantification of mixtures of sheep and human RBCs was based on size differences as disclosed by an impedance cell counter (Coulter Electronics) equipped with a 100 channel size analyzer interfaced with an Apple II+ computer. In some experiments agglutination hindered use of this method, and visible spectroscopy was used to measure hemoglobin concentrations and thus cell numbers. For this procedure, partitioning samples were centrifuged at 1000 *g* for 10 min, the supernatant removed, and 0.4 ml cyanomethemoglobin reagent (Hycel, Houston, TX, U.S.A.) was added to lyse the cells. After centrifugation to remove cell stroma (8800 *g* for 10 min), absorbance measurements were taken at 540 nm and compared to the absorbance of a sample, treated identically, containing a known number of cells.

Direct hemagglutination assay

The method for passive hemagglutination described by Amman and Pelger[20]

was modified to directly assay the agglutinating ability of both PEG-Ab and native antibody fractions. Human and sheep RBCs (between 1 and 7 days old) were collected and washed as described above. Packed, washed cells (0.05 ml) were resuspended in 3.0 ml of solution I [100 ml 0.01 *M* phosphate buffered saline, pH 7.2, 1.6 ml 30% bovine serum albumin (BSA), and 100 mg dextrose]. This suspension could be kept for up to 5 days at 4°C. Before testing, a further 1:7 dilution in solution I was made.

In V-bottom microtiter plates (Cooke Enginnering, Alexandria, VA, U.S.A.) serial two-fold dilutions (0.05 ml) of each sample to be tested were made using solution II (100 ml 0.01 *M* phosphate buffered saline, pH 7.2, 0.05 ml Tween 80, 1.0 mg polyvinylpyrrolidone, and 1.5 ml 30% BSA). Human or sheep RBCs (0.05 ml) were added to each well.

Microtiter plates were placed on an automatic rotator shelf for mixing (10 min) and then incubated on a flat surface overnight at 25°C. Prior to screening, plates were elevated at a 60° angle for 15 min. Positive agglutination resulted in no "run-down" pattern of RBCs. Results were expressed as the minimum concentration of antibody which caused appreciable agglutination.

Preparation of two-phase systems

Polymer phase systems were prepared as described previously[2,3,5,6] by mixing appropriate weights of the following aqueous stock solutions (all %, w/w): 20% dextran T500 (Pharmacia, Piscataway, NJ, U.S.A., lot IE32126 or lot HD26066), 30% PEG 8000 (Union Carbide, lot B529-9104 or lot B-739), 20% dextran T40 (Pharmacia, lot FL18974), 0.6 *M* NaCl, and 0.22 *M* Na_2HPO_4, 0.07 *M* NaH_2PO_4, pH 7.2 buffer. Sodium azide was added to a final system concentration of 0.02% to retard bacterial contamination. Concentrations of dextran solutions were determined by polarimetry[1–3]. All other concentrations were determined gravimetrically. After preparation, phase systems were sterilized by filtration through a 0.2-μm filter and allowed to settle at room temperature overnight. The phases were then separated and stored at 4°C but returned to room temperature before use.

Seven well characterized two-phase polymer systems were used in this work[2,5,21]: (a) (5, 4) I, 5% dextran T500, 4% PEG 8000, 109 m*M* Na_2HPO_4, 35 m*M* NaH_2PO_4, pH 7.2 (buffer I); (b) (5, 4) II, 5% dextran T500, 4% PEG 8000, 50 m*M* NaCl, 73 m*M* Na_2HPO_4, 23 m*M* NaH_2PO_4, pH 7.2 (buffer II); (c) (5, 3.5) V, 5% dextran T500, 3.5% PEG 8000, 150 m*M* NaCl, 7.3 m*M* Na_2HPO_4, 2.3 m*M* NaH_2PO_4, pH 7.2 (buffer V); (d) (4.6, 3.9) V, 4.6% dextran T500, 3.9% PEG 8000, buffer V; (e) (5, 4) V, 5% dextran T500, 4% PEG 8000, buffer V; (f) (7, 4) V, 7% dextran T500, 4% PEG 8000, buffer V; (g) (7, 5) V, 7% dextran T40, 5% PEG 8000, buffer V.

These systems were chosen to represent a range of interfacial tensions, electrostatic bulk phase potentials, and dextran molecular weight[1–5,21].

Single tube partition

The ability of various concentrations of PEG-Ab to increase the partition coefficient *K* (the ratio of cells in the top phase to the remainder of cells) of human RBCs was studied in single-tube experiments. In 75 × 12 mm tubes, 0.5 ml upper phase containing $2 \cdot 10^7$ human RBCs was incubated with 0.2 ml PEG-Ab solution for 15

min at 37°C. The cells were pelleted by centrifugation at 1000 *g* for 10 min and the supernatant was removed. After being washed once in fresh upper phase, cells were resuspended in 1.0 ml upper and 1.0 ml lower phase. The suspension was mixed by inversion twenty times and allowed to settle 15 min for equilibration. Mixing was repeated, the system allowed to settle for 15 min and 0.7 ml of top phase was then promptly removed. A portion of this sample (0.2 ml) was used for impedance counting and the remaining 0.5 ml was centrifuged and quantified by spectrophotometric analysis, as described above.

Inhibition studies

Prior to single tube partitioning, 0.5 ml human RBCs at a concentration of $4 \cdot 10^7$/ml in upper phase was first incubated at 37°C for 15 min with 0.2 ml of unmodified Fab. Cells were then washed once in fresh upper phase and resuspended in 0.5 ml upper phase for incubation with PEG-Ab.

Antibody partition

Both PEG-Ab and native antibody preparations (0.2 ml) at a concentration of 2 mg/ml were added to two ml of a (5, 4.25) V system to produce a (4.9, 3.6) V system. Each tube was mixed twenty times by inversion and allowed to equilibrate for 15 min. After remixing, the systems were centrifuged at 1000 *g* for 5 min. An aliquot of the resulting upper phase was mixed with an equal volume of buffer and its absorbance measured at 280 nm. This value was compared to that of the 100%-control tube in which 0.2 ml of the original sample was added to 0.9 ml of upper phase and 1.1 ml buffer.

BSA partition

Comparison of the partition of unmodified bovine serum albumin (BSA) and PEG-modified BSA was carried out in a (5, 4) V system as follows: preparations (0.1 ml) at a concentration of 4 mg/ml were added to 2 ml of phase system. Tubes were mixed by vortexing twice at 10-min intervals, and were then centrifuged 5 min at 200 *g*. Aliquots from each phase (0.7 ml) were diluted to 3 ml with phosphate-buffered saline (PBS) and fluorescence excitement and monitoring were done at 280 nm and 380 nm, respectively, in a Turner spectrofluorometer with the appropriate phase acting as a zero control.

Automated countercurrent distribution experiments

Countercurrent distribution (CCD) experiments were performed as previously described[1–3,5,6], using a 60-chamber Biosheff MK II apparatus (Biochemistry Department, University of Sheffield, Sheffield, U.K.). The lower cavity volume for this machine is 850 μl. Since all runs described in this work consisted of 30 transfers, either duplicate or two different experiments could be run simultaneously, one on either side of the rotor.

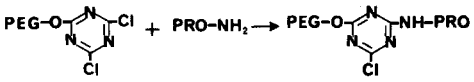
Antibody treatment of RBCs consisted of incubating cells or mixtures of cells with PEG-Ab (1.3 mg/ml) at 37°C for 15 min in the same proportions as for the single tube partitioning experiments. After incubation, cells were washed once with fresh upper phase and resuspended to a load mix concentration of $2 \cdot 10^7$–$4 \cdot 10^7$ cells per ml of upper phase. For partitioning of cells, 800 μl of lower phase was added

to each of the 60 cavities. A volume of 900 μl of sample load mix was added to cavities 0, 1, 30 and 31. A volume of 900 μl of upper phase alone was added to the remaining cavities. In every run, a mixing time of 12 s and a settling time of 3 min was used. Phases were broken by adding 1.0 ml of buffer to each cavity. Cells were quantified as described above.

RESULTS AND DISCUSSION

Antibody modification

Of the several methods available for protein-PEG coupling[22], we chose the method of Abuchowski and Davis[17] in which the PEG is first activated with cyanuric chloride and then coupled to lysine groups on the protein, eqn. 1[18,23].



(1)

Using this reaction we could vary the number of lysines substituted with PEG (as shown by reduction in the number of primary lysine amino groups).

The effect of PEG substitution on protein partitioning in a polymer two-phase system was first tested with BSA, Table I. As can be seen from the shifts in BSA partitioning, attaching PEG to a protein can have a dramatic effect on protein partitioning. Similarly, antibody partitioning can also be shifted to favor the top phase, although partitioning shifts are not as dramatic as with BSA, Table I. Nonetheless, modified antibody was found to strongly favor the top phase, indicating that the proposed affinity partitioning was feasible and that the partition behavior of high MW molecules could be altered by PEG coupling.

Earlier works[14,23,24], especially those of Abuchowski and Davis[17] have amply demonstrated that PEG-substituted enzymes remain active although the loss of activity resulting from substitution can be severe with some enzymes and with some

TABLE I

EFFECTS OF PEG SUBSTITUTION ON BSA AND NATIVE ANTIBODY PARTITIONING IN DEXTRAN–PEG TWO-PHASE SYSTEMS

Protein modified	*Lysines modified (%)*	*Partition (%)****
BSA*	0	31 ± 3
BSA	10	89 ± 3
BSA	39	96 ± 1
BSA	47	96 ± 1
BSA	76	98
Native-Ab**	0	50 ± 3
Native-Ab	47	87 ± 3

* Bovine serum albumin in a (5, 4) V system, dextran T500, PEG 8000; see Materials and methods.

** Sheep IgG anti-human RBCs in a (4.6, 3.9) V system; dextran T500, PEG 8000; see Materials and methods.

*** Percentage of total protein partitioning into the upper phase; BSA ($\bar{x}$ ± SD, n = 2); native-Ab ($\bar{x}$ ± SD, n = 4).

TABLE II

EFFECTS OF PEG SUBSTITUTION ON ABILITY OF ANTIBODY TO AGGLUTINATE RBCs

*PEG $\bar{M}_w$**	*Lysines modified (%)*	*Minimal hemagglutination concentration (μg/ml)***
Control	0	<0.2
(A) 5000	27	23 ± 8
(B) 5000	40	19 ± 8
(C) 5000	51	5 ± 2
(D) 1900	45	1 ± 0
(E) 1900	70	3 ± 1

* Molecular weight of PEG used for substitution of sheep IgG anti-human RBCs. Letter designation refers to Fig. 1.

** Lowest antibody concentration exhibiting appreciable hemagglutination in microtiter assay ($\bar{x}$ ± SD, n = 3).

coupling methods. The present study is the first to our knowledge utilizing PEG-substituted antibody[25]. As shown in Table II, PEG-Ab loses some of its ability to agglutinate human RBCs. This effect shows an apparent dependence on PEG mol.wt., the higher mol.wt. causing more deactivation. The effect of percentage substitution on antibody activity is not a simple relationship since the PEG 5000 is more active with a higher percentage substitution while the PEG 1900 is less active, Table II. However, measurements of agglutination and percentage substitution are sufficiently approximate to make this latter conclusion uncertain. We are presently using more precise techniques to examine the effects of PEG substitution on enzyme activ-

TABLE III

PARTITIONING OF HUMAN RBCs AS A FUNCTION OF PEG-Ab CONCENTRATION AND DEGREE OF ANTIBODY MODIFICATION

*Modification** *(%, MW, origin)*	*Partition (%)***					
	[PEG-Ab] (μg/ml)					
	5.35	*10.7*	*21.4*	*42.8*	*85.7*	*171*
27% 5000 SHE (A)	47	46	49	53	79	92
40% 5000 SHE (B)	33	39	48	56	77	84
50% 5000 SHE (C)	36	38	38	45	54	80
45% 1900 SHE (D)	41	34	39	44	55	57
70% 1900 SHE (E)	40	45	46	47	54	61
47% 5000 RAB***	3.0	3.0	4.0	12	35	80

* Given as lysines modified (%) by PEG; MW of PEG used; and origin (sheep or rabbit) of antibody; A–E designation refers to Fig. 1.

** Duplicate determinations with average standard deviation of 2.4%. Partitioning was done in separate batches of (4.6, 3.9) V phase system (dextran T500, PEG 8000; see Materials and methods). In absence of PEG-Ab 2% partition of human RBCs was observed in the rabbit experiments and 35% in the sheep experiments (see Discussion).

*** Additional values of [PEG-Ab]/% partition: 322/93; 643/90; 1286/84; and 2200/83.

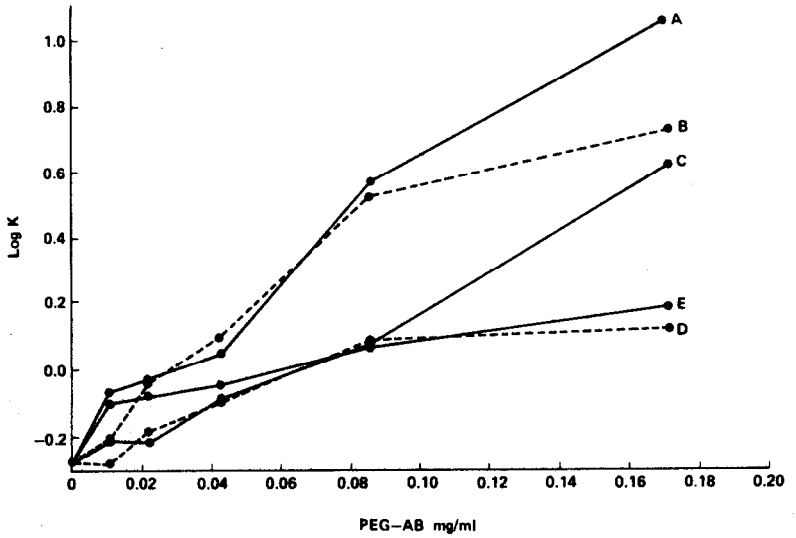


Fig. 1. Effects of PEG molecular weight, extent of modification, and incubation concentration of PEG-Ab (derived from sheep) on the upper-phase affinity (log K) of human RBC. K is defined as the ratio of the number of cells in the top phase to the remainder. (A) PEG 5000, 27% modification; (B) PEG 5000, 40% modification; (C) PEG 5000, 51% modification; (D) PEG 1900, 45% modification; (E) PEG 1900, 70% modification.

ity. For the present study, however, these effects are not of critical importance, since all that is needed is PEG-substituted antibody which is active and partitions to the top phase. In a control experiment PEG-Ab (antibody from sheep) was shown to have no agglutinating activity toward sheep RBCs.

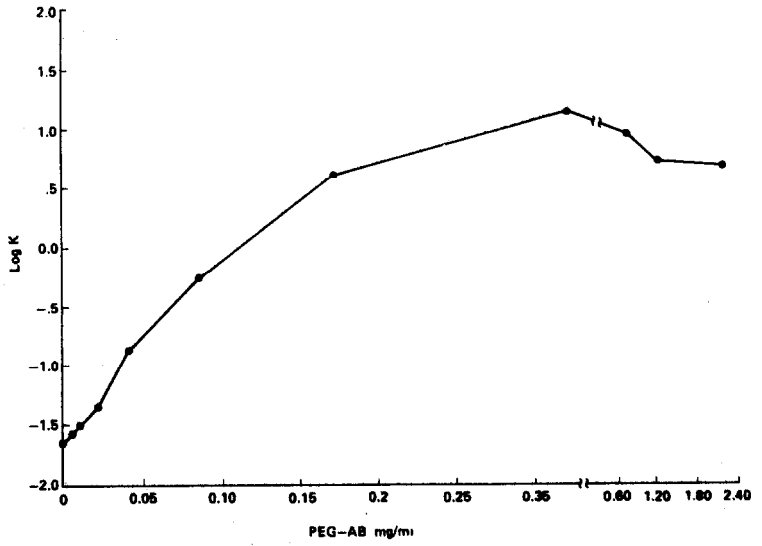


Fig. 2. Effect of incubation concentration of PEG-Ab (derived from rabbit) on the upper-phase affinity (log K) of human RBC.

Single tube partition

Next the ability of the PEG-Ab to alter the partitioning of human RBCs was examined. These results are shown in Table III and Figs. 1 and 2. As we had hoped, the PEG-modified antibody is quite effective at pulling human RBCs into the top phase; the increase in partition varies directly with the amount of PEG-Ab added. Note also that the partitioning shift is observed with both sheep- and rabbit-derived antibody. Unmodified antibody did not alter RBC partition, either because of extensive agglutination and sedimentation or because PEG is necessary to pull the cells into the PEG-rich phase.

Antibody modified with PEG 5000 gives more dramatic effects, especially evident at the higher concentrations of modified antibody; for example, at 171 μg/ml the PEG-5000 conjugate gave an average partition of 85% while the PEG 1900 conjugate gave an average partition of 59%. Interestingly, the least agglutinating PEG-Ab gave the highest percentage partition; with the more active antibody, agglutination and sedimentation of cell clumps was a problem. Thus it appears that the reduction in antibody agglutinating ability which results upon PEG coupling is advantageous from the standpoint of affinity partitioning.

Finally, it remains to demonstrate that the PEG-Ab is selective in pulling only specific cells into the top phase. Single-tube control experiments clearly showed that partitioning of sheep RBCs was unaffected by PEG-modified antibody. Thus all the components of immuno-affinity phase partitioning of cells are in hand: PEG-modified antibodies will dramatically and selectively shift the partitioning of the targeted cells into the top PEG-rich phase.

In a related study, Sharp *et al.*[25] have shown that human RBCs bind approximately 15 000 PEG-Ab molecules of rabbit-derived anti-human antibody. This study indicates that the present technique may be applicable to cells having this approximate receptor density.

As an additional control experiment, we also examined the ability of monovalent Fab fragments prepared from the same lot of rabbit IgG anti-human RBC (unmodified by PEG attachment) to inhibit the shift in human RBC partitioning that results in the presence of PEG-modified antibody. For this experiment the following concentrations were used: Fab, 686 μg/ml; 47% modified PEG 5000 IgG, 600 μg/ml; and $2 \cdot 10^7$ human RBCs in 2 ml of a (4.6/3.9) phase system. In the absence of Fab 89% of the RBCs partitioned to the top phase. Incubation of the cells with Fab prior to their incubation with PEG-Ab gave a reduction in partitioning to 69% (expected from 150 μg of PEG-Ab). Unfortunately, this experiment was confused somewhat by unexpected cell aggregation by the Fab (which surprisingly, occurred in phase system but not in buffer). Nonetheless, Fab did compete with the PEG-modified IgG in binding with the RBCs and thus gave a reduction in the affinity partitioning effect.

System variation

After these initial experiments, several other phase systems were examined to determine the effects of various phase system parameters on affinity partitioning, Table IV. In brief, we observed that PEG-Ab addition produced the most dramatic shifts in cell partition in systems having a low interfacial tension and a low electrostatic bulk phase potential. As would be expected, increasing the interfacial tension pulled the cells to the interface, making it more difficult to pull the cells into either phase [*e.g.*, compare the (5, 3.5) V, (5, 4) V, and (7, 4) V systems].

TABLE IV

EFFECT OF PHASE SYSTEM COMPOSITION ON ABILITY OF PEG-Ab TO INDUCE ALTERATION OF RBC PARTITION

*Two-phase system**	*Interfacial tension (μN/m)***	*Electrostatic bulk phase potential (mV)***	*Cell location****	*Partition (%)*§	
				No PEG-Ab	*PEG-Ab*
(5, 4) I	11.0	2.2	Upper	14 ± 1	58 ± 4
(5, 4) II	8	1.0	Upper	6 ± 1	64 ± 1
(5, 3.5) V	0.7	0.2	Upper	2 ± 1	77 ± 1
(4.6, 3.9) V	3	0.2	Upper	2 ± 1	69 ± 3
(5, 4) V	4.9	0.3	Upper	0 ± 1	30 ± 1
(7, 4) V	17.2	0.2	Upper	0 ± 1	4 ± 2
(7, 5) V*	5.0	0.2	Upper	0 ± 1	1 ± 1
			Interface	45 ± 3	97 ± 3
			Lower	55 ± 3	3 ± 1

* All systems compounded with PEG 8000 and dextran T500 of the same lots except (7, 5) V which was prepared with dextran T40; see Materials and methods.

** Taken from ref. 21.

*** Referring to the upper or lower phase or to the interface between them.

§ Mean ± S.D. of two independent determinations using 643 μg/ml of PEG-Ab (from rabbit).

The effects of electrostatic bulk phase potential are difficult to determine since increasing potential (by salt variation) is accompanied by an increase in interfacial tension (*e.g.*, compare the I, II, and V versions of the 5, 4 system). Generally, however, it appears that increasing the potential is counterproductive for affinity partitioning since it increases the partition coefficient K (ratio of cells in top to the remainder of cells) in the absence of affinity ligand. Also, the high interfacial tension for high potential systems [*e.g.*, (5, 4) I] makes it difficult for the affinity ligand to pull cells away from the interface; note the decrease in partition coefficient in the presence of ligand upon moving from V to II to I versions of the (5, 4) system. The net result is that the largest shifts in partitioning from ligand addition are found with the low tension systems such as the (5, 3.5) V system.

The final entry in Table IV is for a (7, 5) V system made from dextran of molecular weight 40 000. The lower dextran molecular weight, as expected[1,2], gives a lower partition coefficient, with a significant percentage of the cells moving to the

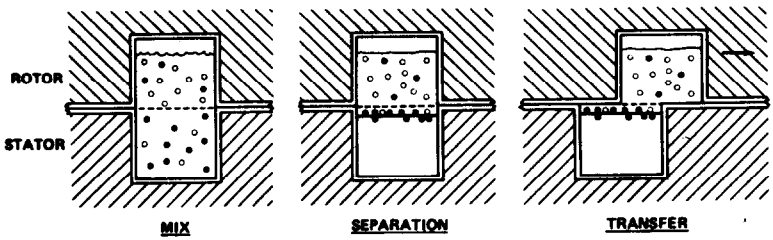


Fig. 3. Thin-layer countercurrent distribution (CCD) in aqueous polymer two-phase systems. The phases are mixed, allowed to separate, and material distributed between cavities is separated by transferring the upper cavity onto a fresh lower cavity. This process can be repeated (from refs. 5 and 6).

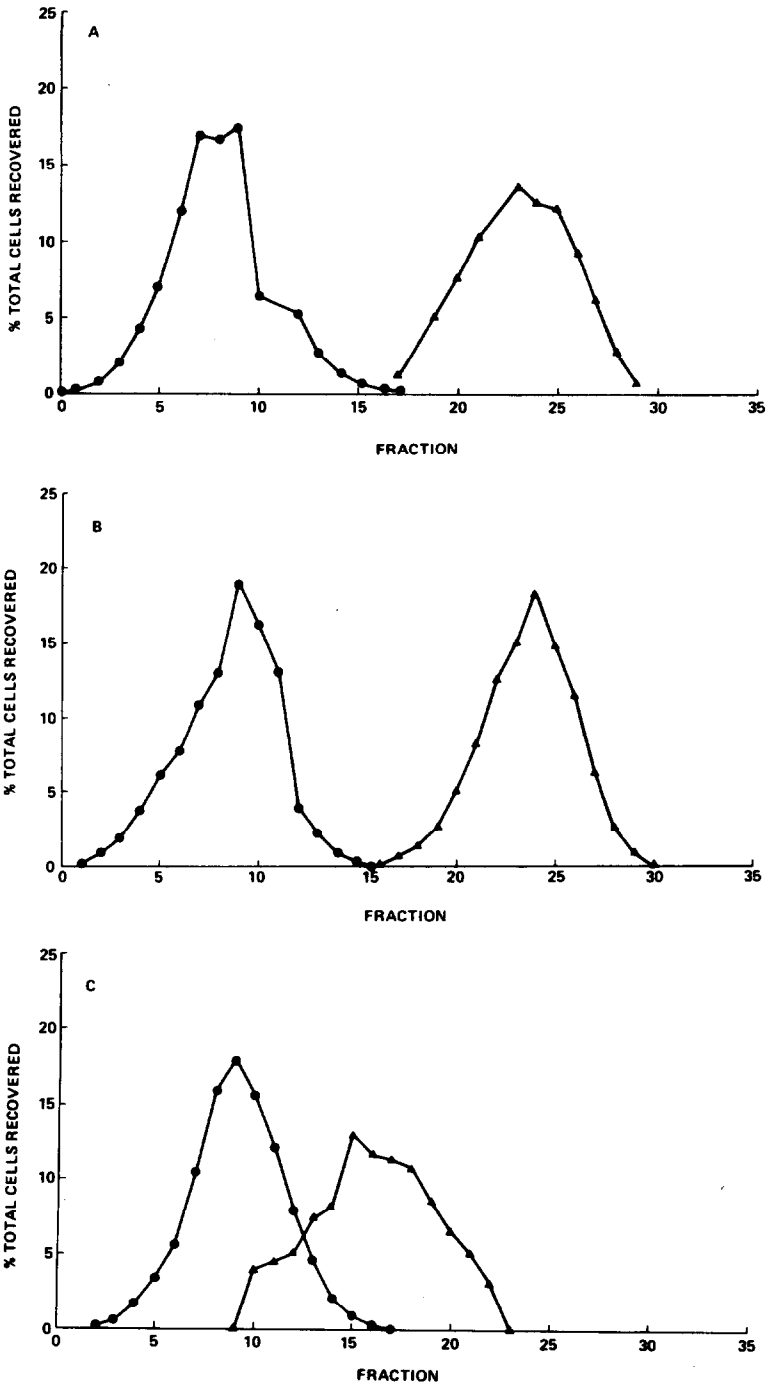


Fig. 4. Superimposed 30-transfer CCD runs of human RBC in a (4.6, 3.9) V two phase system. (A) RBC incubated with 1.3 mg/ml PEG-Ab (30% modification) and washed prior to CCD (▲); control RBC incubated in buffer only and washed prior to CCD (●). (B) Identical to A except a different preparation of PEG-Ab used for incubation (47% modification). (C) Identical to B except that an incubation concentration of 0.325 mg/ml was used.

lower, dextran-rich phase. This system appears to be of little use for affinity partitioning since the ligand acts to move cells from the bottom phase to the interface, which already contains a large number of the cells. However, it is noteworthy that the PEG-Ab binding is powerful enough to pull the cells from the bottom phase.

Finally, it should be noted that the (4.6, 3.9) V entry (without ligand) for Table IV is quite different from the same entry for Table III. These two results differ because different lots of PEG 8000 were used. It is not uncommon in partitioning to observe this sort of unexplained dependence on polymer lots[21].

Countercurrent distribution

The shifts in partitioning produced by adding PEG-Ab are not large enough to produce total separation in one step. This is a familiar situation in phase partitioning with aqueous polymer two-phase systems, and the usual approach is to utilize an automated device which provides repeated partitioning[1–7]. In the present case we have performed 30-transfer CCD experiments with a Biosheff MK2 apparatus. In a CCD device the cells are mixed with the two-phase system in one cavity, then the top phase is shifted to the adjacent bottom phase and the bottom phase is shifted to the adjacent top phase, Fig. 3. These affinity CCD experiments could be performed in two ways. Either PEG-Ab could be added to every cavity, in which case a large quantity of PEG-Ab would be required, or the cells could be incubated once with the PEG-Ab prior to CCD; in this latter case little PEG-ligand would be required.

In Fig. 4 we have presented the results of three CCD experiments. In the first, Fig. 4a, untreated human RBCs (circles) and human RBCs previously incubated with PEG-Ab for 15 min at 37°C (triangles) were subjected to separate 30-transfer CCD runs. As would be expected on the basis of the higher partition coefficient for human RBCs in the presence of PEG-Ab (from single tube experiments), the human RBCs incubated with PEG-Ab gave a peak well separated from that of the untreated human RBCs. Fortunately, the incubation technique provided sufficient exposure of the RBCs to antibody, thus dramatically reducing the amount of PEG-Ab required to perform CCD experiments.

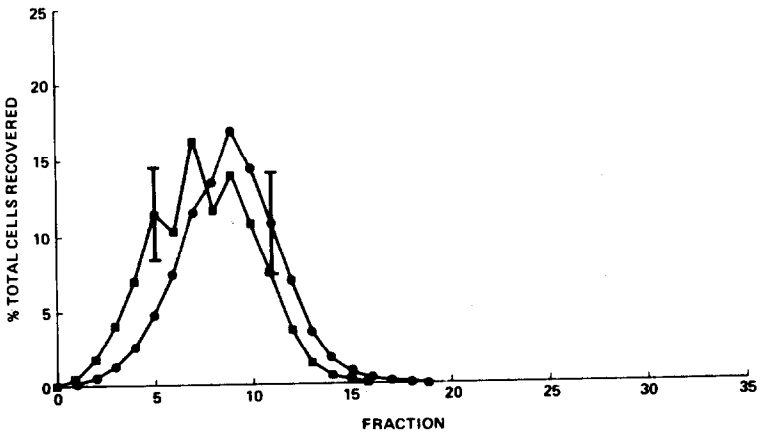


Fig. 5. Superimposed 30-transfer CCD runs of human (●) and sheep (■) RBCs in a (4.6, 3.9) V two-phase system. Results are averages of 4 runs for human and 7 runs for sheep.

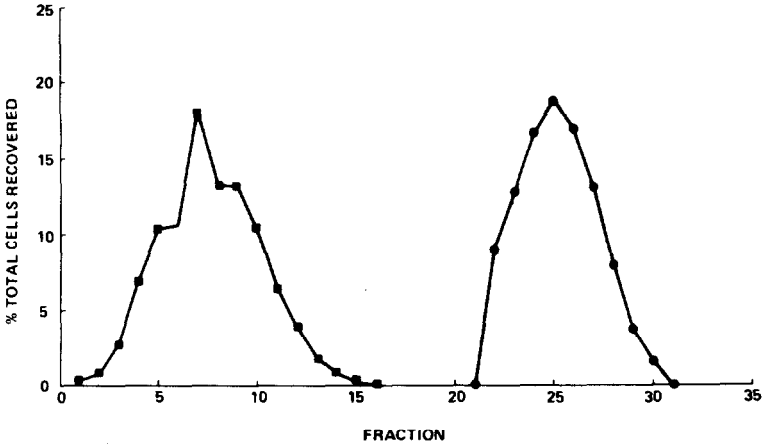


Fig. 6. 30-transfer CCD of a mixture of $7 \cdot 10^7$ human (●) and sheep (■) RBCs in a (4.6, 3.9) V two-phase system following incubation with 1.3 mg/ml PEB-Ab (from rabbit) and washing with phase system to remove unadsorbed antibody.

The experiment of Fig. 4a was performed with rabbit-derived PEG-Ab with 30% of the lysines substituted by PEG. This particular PEG-Ab sample gave extensive cell agglutination. To avoid cell sedimentation in this case we used a large excess of PEG-Ab in our incubation procedure to lessen the likelihood of a particular antibody encountering more than one cell. Interestingly, not all PEG-Ab preparations gave this same behavior. For example in Fig. 4c we show a CCD curve for untreated and treated human RBCs in which incubation was done with a relatively small amount of PEG-Ab in which the IgG was rabbit-derived and had 47% of its lysines substituted with PEG. In this case an antibody excess was not required because cell sedimentation was minimal. The poor separation of Fig. 4c results apparently because insufficient PEG-Ab is bound to the human cells. In Fig. 4b we show CCD curves for the weakly agglutinating antibody of Fig. 4c but at the same concentration as in Fig. 4a; nearly identical results are obtained.

These results emphasize that appreciable variation in PEG-Ab preparations was found. On the negative side, we must say that the origin of this variation is unclear, while on the positive side, we can note that all the variants were effective in immuno-affinity cell partitioning.

Prior to attempts to separate mixtures of human and sheep RBCs, control experiments were done, Fig. 5. In these experiments, sheep and human RBCs which were not incubated with PEG-Ab revealed identical CCD behavior, with both RBCs peaking in early fractions.

In our final experiment, shown in Fig. 6, sheep and human RBCs were mixed, incubated with PEG-Ab, then subjected to a 30-transfer CCD run. Again, the human and sheep RBCs were cleanly separated, with the human RBC's moving far down the CCD train.

CONCLUSION

In summary, we have demonstrated that immuno-affinity phase partitioning

with PEG-modified antibodies as affinity ligands is an effective technique for cell purification in an erythrocyte model system. This advance might reasonably be expected to be applicable to cell separations of significant biomedical interest. Work is in progress in our laboratories to explore this prospect.

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