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CELL SEPARATION BY IMMUNOAFFINITY PARTITIONING WITH POLYETHYLENE GLYCOL-MODIFIED PROTEIN A IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

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SUMMARY

Previous work has shown that polyethylene glycol (PEG)-bound antibodies can be used as affinity ligands in PEG–dextran two-phase systems to provide selective partitioning of cells to the PEG-rich phase. In the present work we show that immunoaffinity partitioning can be simplified by use of PEG-modified Protein A which complexes with unmodified antibody and cells and shifts their partitioning into the PEG-rich phase, thus eliminating the need to prepare a PEG-modified antibody for each cell type. In addition, we provide a more rigorous test of the original technique with PEG-bound antibodies by showing that it is effective at shifting the partitioning of either cell type of a mixture of two cell populations.

INTRODUCTION

Aqueous solutions of low concentrations of the polymers dextran and polyethylene 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 these two-phase systems can be used for analytical and preparative separations of a wide range of biological materials by differential partitioning between the two phases or, in the case of particles, between the liquid–liquid interface and either of the phases[1–4]. When differences in partition are great enough, adequate separations can be obtained in a single tube. Often this is not the case, however, and resolution can then be enhanced through the repeated partitioning provided by counter-current distribution (CCD) and counter-current chromatography[1–6].

Partitioning differences can also be improved by use of affinity ligands attached to one of the polymers (generally PEG)[6–16]. Recently, we[15] and Sharp *et al.*[16] demonstrated that PEG-modified antibodies can be used to substantially and specifically shift the partitioning of target cells from the phase interface to the PEG-rich phase. In the present work we show that immunoaffinity partitioning can be simpli-

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fied by use of PEG-modified Staphylococcal Protein A (PEG–SpA) to bind with antibody–cell complexes and shift cell partitioning to the PEG-rich phase, thus eliminating the need to prepare a separate PEG-modified antibody for each cell type.

The tendency for SpA to bind selectively to immunoglobulins, particularly to immunoglobulin G (IgG), was first noted by Verwey in 1940[17]. Since then, SpA has proved useful in a wide variety of immunological methods (for review, see refs. 18 and 19). Ghetie *et al.*[20] have used SpA bound to Sephadex for enrichment of cell populations by column chromatography. It has also been used to coat glass beads[21] and iron dextran microspheres[22] for the isolation and quantification of antibodies, antigens and cells. Ling and Mattiasson[23] have described the use of PEG-modified *Staphlyococcus aureus* to increase the partition of antibody and thus separate bound from unbound antigen in a radioimmunoassay for B_2 macroglobulin using a two-phase polymer system.

In previous work[15] we used PEG-modified IgG antibody preparations directed against human red blood cells (RBCs) to shift the partitioning of the human RBCs into the PEG-rich phase and to completely separate a mixture of human and sheep RBCs by 30-transfer CCD. However, it is known that decreasing the interfacial tension in polymer phase systems by reducing polymer concentration will also cause this same shift in partitioning of human erythrocytes[3]; a similar partitioning shift for sheep erythrocytes does not normally result from this slight modification of the phase system. In the work presented here we provide a more rigorous demonstration of immunoaffinity cell separation using PEG-modified antibody preparations directed against sheep RBCs to shift the partitioning of these cells into the top phase and leave the human cells at the interface.

MATERIALS AND METHODS

Protein preparations

Rabbit IgG directed against sheep or human RBCs was obtained from Cooper Biomedical (Malvern, PA, U.S.A.) and used without further purification. PEG-modified antibody (PEG–Ab) was prepared as described previously[15,24], by activation of PEG-5000 monomethyl ether (Union Carbide, Tarrytown, NY, U.S.A.) with cyanuric chloride, followed by coupling of the activated PEG to the protein. Analysis of the modified protein with trinitrobenzenesulfonic acid[15] showed that 40% of the protein lysine groups had been modified. Protein A (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) was modified (PEG–SpA) and analyzed similarly; 60% of lysine groups were covalently bound to PEG. Preparations were further analyzed by gel filtration through Superose 12 (Pharmacia Fine Chemicals). As expected, PEG-modified preparations yielded distinct peaks which indicated substances of much larger molecular weight than the unmodified preparations.

RBC preparation

Human RBCs were obtained by venipuncture from healthy individuals, treated with 10 m*M* EDTA as an anticoagulant and stored at 4°C. Sheep RBCs were purchased from Cooper Biomedical and stored at 4°C. Shortly before use, 1-ml aliquots were washed four times by suspension in twenty volumes of 0.01 *M* phosphate buffered saline, pH 7.4, followed by centrifugation for 10 min at 1000 *g* and removal of

the supernatant and the buffy coat. Quantification of mixtures of sheep and human RBCs was based on size difference, as disclosed by an impedance cell counter (Coulter Electronics, Hialeah, FL, U.S.A.) equipped with a 100-channel size analyzer interfaced with an Apple II$^+$ computer.

Preparation of two-phase systems

Polymer phase systems were prepared as described previously[15] by mixing appropriate amounts of PEG-8000 (Union Carbide, lot B529-9104), dextran T500 (Pharmacia, lot IE32126), and buffer. Distilled, filtered 12 MΩ/cm water was used throughout. Experiments were performed with a system containing 4.6% (w/w) dextran, 3.9% PEG, and 0.02% sodium azide in 0.15 *M* sodium chloride, 0.01 *M* sodium phosphate buffer (pH 6.9). This system was chosen to provide a direct comparison with our previous study[15]. Moreover, untreated sheep and human RBCs tend to partition to the interface in this system. Since the percentages of dextran and PEG are close to the critical concentrations where separate phases no longer form, the interfacial tension is very low, and cells are easily moved to the upper phase by treatment with PEG-bound affinity ligands[15].

Single-tube partition

Antibody preparations that have been modified with PEG remain active (bind to cell surface antigen)[25] but lose much of the tendency to cause cell agglutination[15]. Since the immunoaffinity procedure using PEG–SpA utilizes unmodified antibody, care must be exercised to avoid cell agglutination during incubation. Single-tube experiments were performed to qualitatively determine parameters, such as concentration of reagents and their order of addition, as well as length of time and temperature of incubation, which best combine to yield a minimum of cell agglutination with a maximum shift in partition to the upper, PEG-rich phase.

In a typical single-tube experiment, cells were incubated with PEG–SpA and the appropriate unmodified antibody preparation (in plastic 75 × 12 mm tubes). Cells were washed once with fresh upper phase, and resuspended in 1.0 ml of upper and 1.0 ml of 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 again for 15 min, and a portion from the center of the upper phase was immediately removed and used for impedance counting and for examination for cell agglutination.

Automated CCD experiments

CCD experiments were performed as described previously[15] using a 60-chamber Biosheff MK 2N apparatus (Biochemistry Department, University of Sheffield, Sheffield, U.K.). The lower cavity volume for this machine is 850 μl. All runs described in this work consisted of 30 transfers.

PEG–Ab treatment of RBCs consisted of incubating mixtures of cells suspended in upper phase (at a concentration of $4 \cdot 10^7$ cells per ml) with PEG–Ab (at a concentration of 1.5 mg/ml) for 15 min at 37°C. Cells were centrifuged at 1000 *g* for 5 min, washed once with fresh upper phase, and resuspended to a concentration of $(1.5–2.0) \cdot 10^7$ cells per ml in upper phase. Lower phase (800 μl) was added to each of the 30 cavities. A volume of 900 μl of the cell sample was added to cavities 0 and

1, and a volume of 900 μl of upper phase was added to the remaining cavities. A mixing time of 12 s and a settling time of 3 min were used. Cells were quantified as described above.

Protein A experiments were performed similarly with the exception that cells were first incubated with PEG–SpA and unmodified Ab as follows. Mixtures of cells [(3–4) $\cdot$ 10^7 total cells] suspended in upper phase were centrifuged at 1000 *g* for 5 min. The supernatant was removed and resuspension of cells in the remaining liquid was achieved by gentle vortexing. PEG–SpA (0.33 ml) was first mixed with the cells, followed by addition of an equal volume of unmodified antibody and incubation for 60 min at room temperature with occasional agitation. Cells were then washed once with fresh upper phase and resuspended to a concentration of (1.5–2.0) $\cdot$ 10^7 cells per ml. Incubation concentrations of PEG–SpA, unmodified anti-sheep RBCs antibody and unmodified anti-human RBCs antibody were 0.8 mg/ml, 0.08 mg/ml and 0.12 mg/ml, respectively.

RESULTS AND DISCUSSION

Separation with PEG-modified antibody

As noted in our introductory comments, the same partitioning shifts observed with human RBCs treated with PEG–Ab can also be produced by slight changes in the phase system, although it is quite difficult to produce a similar shift in partitioning of sheep RBCs[3]. Thus, a more demanding test of the PEG–Ab approach to immunoaffinity partitioning is to modify antibody directed against sheep RBCs with PEG and show that this PEG–Ab will cause a shift in partitioning of sheep RBCs to the PEG-rich phase.

Results of a typical experiment are presented in Fig. 1. A mixture of sheep and human RBCs was incubated with PEG-modified antibody (from rabbits) directed against sheep RBCs and then subjected to 30-transfer CCD in the 4.6, 3.9 phase system. Note that partitioning in this phase system without PEG–Ab incubation is

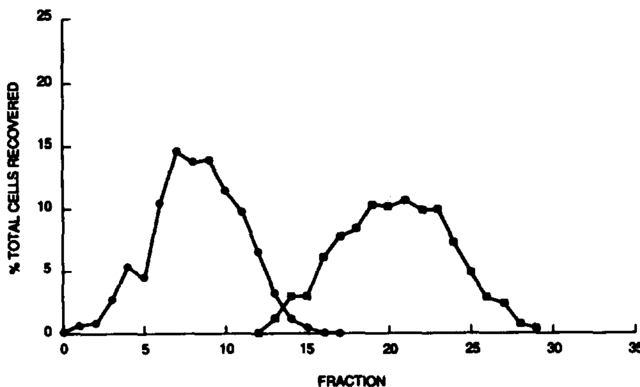

Fig. 1. 30-transfer CCD of a mixture of 3 $\cdot$ 10^7 human (●) and sheep (■) RBCs in a two-phase system consisting of 4.6% dextran T500, 3.9% PEG-8000, and 0.02% sodium azide in 0.15 *M* sodium chloride, 0.01 *M* sodium phosphate buffer (pH 6.9). Prior to CCD, cells were incubated with 1.5 mg/ml PEG–Ab directed against sheep RBCs and washed with upper phase to remove unadsorbed antibody.

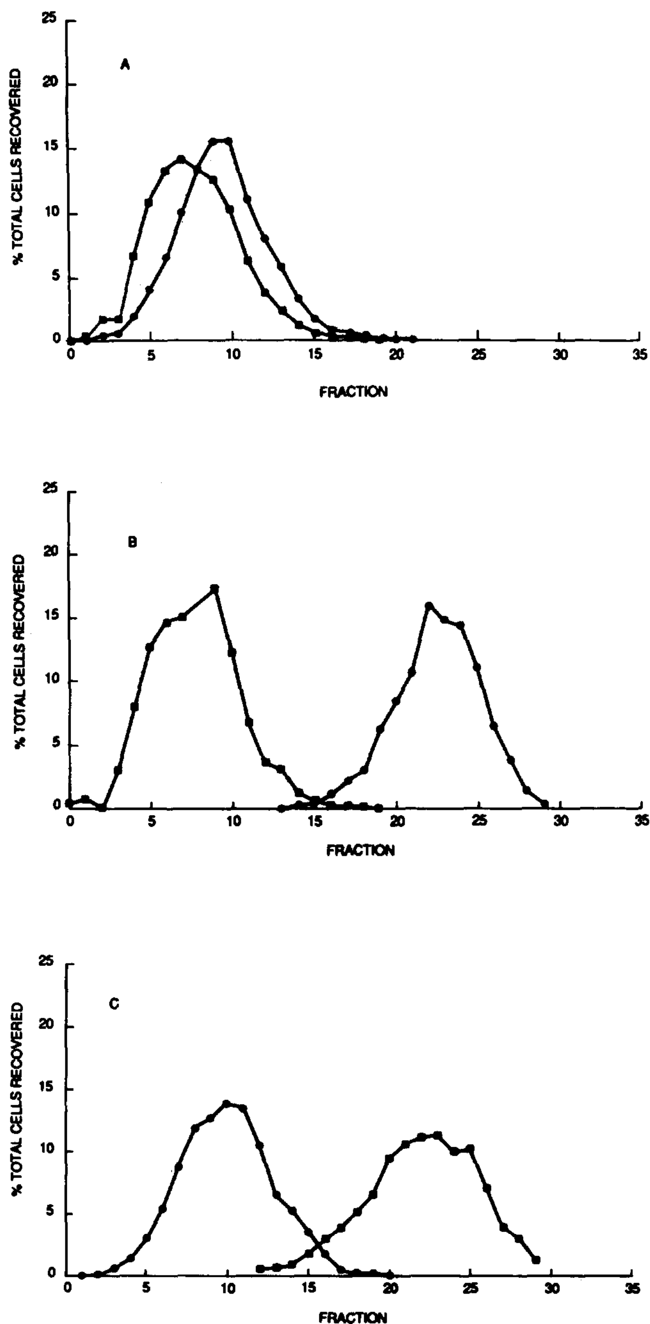

Fig. 2. (A) 30-transfer CCD of a mixture of $3 \cdot 10^7$ untreated human (●) and sheep (■) RBCs. Two-phase system used is the same as in Fig. 1. (B) Identical to A except that prior to CCD, RBC mixture was incubated with 0.8 mg/ml PEG–SpA and 0.12 mg/ml unmodified antibody directed against human RBCs. (C) Identical to A except that prior to CCD, RBC mixture was incubated with 0.8 mg/ml PEG–SpA and 0.08 mg/ml unmodified antibody directed against sheep RBCs.

similar for both human and sheep RBCs, so that CCD produces little separation of the cells (Figure 2A). As shown in Fig. 1, incubation of the cells with the PEG-modified antibody against sheep RBCs leads to a shift in partitioning of the sheep RBCs to the PEG-rich phase and to a nearly complete separation of sheep and human cells. The separation in this case is quite comparable to that observed previously when the cell mixture was incubated with PEG–Ab directed against human RBCs[15]. The present result, showing a shift in partitioning of sheep RBCs, clearly demonstrates that our previous observation of a shift in partitioning of human RBCs after incubation with PEG–Ab directed against human RBCs was the result of binding of the PEG–Ab to the cells and not the result of an unintended alteration in the phase system.

Single-tube partition

Parameters for incubation of cells with PEG–SpA and unmodified Ab prior to CCD were determined from results of qualitative single-tube partition experiments. Several aspects of the incubation process needed to be examined. It was necessary to determine optimum temperature and time for incubation, and it was important to determine the best order of addition of reagents to avoid excessive agglutination of cells. Additionally, variations in the proportions of reagents to each other, as well as to the number of cells incubated, were investigated.

Incubation of cells with PEG–SpA and unmodified Ab was carried out at 0°C, room temperature and 37°C for 15, 30 and 60 min. Although PEG–SpA did not appear to attach well at 0°C (as evidenced by only small shifts in partition), there was little difference between incubation at room temperature and at 37°C. Shifts in partitioning of cells were progressively higher at 30 min and 60 min incubation than at 15 min. Because time is often an important factor when preparing live cells for CCD, incubation times greater than 60 min were not tested.

Adding unmodified antibody to cells prior to addition of PEG–SpA was not practical, as cells were agglutinated even by very dilute antibody preparations. Cell agglutination was seen when cells and antibody dilutions were prepared in either phosphate-buffered saline or in upper phase. At dilutions great enough to avoid agglutination, apparently too few antibody molecules were bound per cell to effect a significant increase in partition.

Another method tested for incubation of cells involved mixing unmodified Ab with PEG–SpA for 60 min at room temperature; after which cells were added and incubation carried out for 15 min at 37°C. This method worked well in single-tube partition experiments but cells later agglutinated during 30-transfer CCD. Although not further pursued, additional experiments using the same order of addition but varying the relative concentrations of antibody and PEG–SpA might prove beneficial.

Best results were obtained, with very little cell agglutination, when PEG–SpA was first mixed with cells (suspended in a small volume of upper phase), followed by immediate addition of unmodified antibody and incubation for 60 min at room temperature with occasional agitation. Untreated human RBCs partitioned an average of 7.5% into the upper phase. However, human RBCs treated as described with PEG–SpA and anti-human RBC antibody partitioned an average of 70% into the upper phase. Similarly, the partitioning of sheep RBCs into the upper phase increased from 3 to 65% upon treatment with PEG–SpA and anti-sheep RBC antibody.

Treatment of human RBCs or sheep RBCs with PEG–SpA alone produced no increase in partition. PEG–SpA partitioned an average of 71% into the top phase while unmodified SpA partitioned only 32%. Treatment of either cell type with its respective unmodified antibody produced extensive agglutination, but no apparent increase in partition.

To obtain good separation with very little cell agglutination, approximately 25–35 times as much PEG–SpA was added as was necessary to bind all IgG molecules on a molar basis. SpA is known to form soluble complexes with rabbit IgG which vary in composition and ability to agglutinate cells depending on the relative proportions of SpA and IgG. At low SpA to IgG ratios, multivalent IgM-like complexes are formed with a formula of $[(IgG)_2SpA]_2$. At high SpA to IgG ratios, each SpA binds only one IgG molecule, and cell agglutination is less likely to occur[26].

Although the incubation parameters described appeared optimum for the RBC models used, it should be emphasized that different conditions may prove necessary for other cell types. Moreover, differences in binding between SpA and IgG from other species or from subclasses within the same species may require adjustments in regard to temperature, buffer pH, time for incubation, or relative concentrations of reagents.

Separations with PEG-modified Protein A

Typical results of our experiments with Protein A are shown in Fig. 2. Fig. 2A shows a control experiment in which a mixture of sheep and human RBCs are subjected to 30-transfer CCD in the 4.6, 3.9 phase system chosen to localize the bulk of both sheep and human RBCs at the phase interface. As can be seen from Fig. 2A, there is little if any separation of the two-cell types after 30-transfer CCD.

The results of incubating cells with antibody and PEG–SpA are shown in Fig. 2B and C. As shown in Fig. 2B, incubating the cell mixture with PEG–SpA and unmodified antibody directed against human RBCs followed by 30-transfer CCD gave two well resolved peaks, with the peak having the higher partition coefficient being human RBCs. Clearly, the PEG–SpA is binding to antibody on the cell surface, which shifts partition of the human RBCs toward the PEG-rich phase. Similarly, incubating the cell mixture with PEG–SpA and antibody against sheep RBCs preferentially shifts sheep RBC partition into the upper phase (Fig. 2C). Comparison of these separations with those provided by direct binding with PEG-modified antibody as discussed above shows that the Protein A approach seems to be just as effective as the PEG–Ab approach to immunoaffinity partitioning of cells, in an RBC model system.

Since the method using PEG–Ab requires preparation of a different PEG-modified antibody for each cell type to be separated, while the PEG–SpA approach does not, a more general procedure is now at hand with the potential for purification of cells for which a specific antibody is available. The method using PEG–SpA requires less than 10% of the antibody concentration as when directly modified antibody is used. With the use of affinity purified and monoclonal antibodies even less antibody and SpA should be required. Limitations of the procedure are that cells which have IgG present as part of their normal surface may be co-purified, and that there must be sufficient antigenic sites on the cell surface to bind enough Ab–PEG–SpA to shift the partition coefficient to the upper phase. Work in progress includes exploration

of the limits of this second factor to determine the minimum number of binding sites required to produce the needed partitioning shift.

Comments on other purification methods

Purification with good yield of viable, normal-functioning cell populations is of great interest in many areas of biomedical science. Several purification methods used recently utilize specific antibodies to cell surface antigens and have been applied with varying degrees of success. (For a recent review of these methods as they apply to purification of lymphocyte subpopulations using monoclonal antibodies, see ref. 27.) Although the fluorescent activated cell sorter has been used with much success for the diagnostic quantification of cell populations, it is not rapid enough for purification of large numbers of cells while maintaining sterility and cell viability. Moreover, the equipment is costly and requires considerable expertise to use.

Procedures using antibody-coated plastic dishes (panning), often have low yields as well, and positively selected preparations may be contaminated with non-adherent populations. Immunoaffinity column chromatography has been used with moderate success for depletion of certain cell types from a mixture, but its use for positive selection of cells is hindered by difficulty in removing attached cells from the column matrix. Levitt and Danen[28] have reported a novel method by which both positively and negatively selected populations are recovered. Cells are coated with biotin-conjugated specific antibody and form rosettes when mixed with advidin-coated erythrocytes. Rosetted and non-rosetted cells are separated by density centrifugation and erythrocytes are then lysed with hypotonic buffer. Recovery of cells is relatively good (50–75%), but a different biotin–antibody conjugate must be prepared for each cell type to be isolated.

CONCLUSIONS

In summary, PEG-modified Protein A can be used in conjunction with IgG antibody to effectively separate RBCs by phase partitioning while achieving virtually complete recovery of apparently undamaged cells. Phase partitioning has long been noted for the gentle environment provided living cells during separation procedures, and with the use of immunoaffinity ligands (both PEG-modified antibodies and PEG-modified Protein A), it should become a powerful method for isolating specific cell populations.

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