Magnetic Microspheres Prepared by Redox Polymerization Used in a Cell Separation Based on Gangliosides

Abstract. *A facile method is described for making magnetic microspheres that bind specifically to cell surfaces, in order to separate cells magnetophoretically. Control over the sizes of the spheres is effected by using their magnetic cores as part of a redox polymerization system. The use of the microspheres is demonstrated with a separation involving C-1300 neuroblastoma cells, 10 percent of which express the ganglioside G_{M1} in their membranes. The G_{M1}-containing cells were separated with better than 99 percent purity, while the deficient cells were obtained at least 98 percent pure. The separation, which was carried out under sterile conditions, required only 6 minutes.*

Investigations of cells and tissues would be greatly facilitated if a cell-separation technique were available that is as convenient and reliable as filtration is in chemistry. In this report we describe a simple technique based on cell-surface characters that approaches this goal, separating a mixture of two types of cells to better than 98 percent purity in minutes.

Separation of cells based on their surface markers has generally involved either specific adherence to fixed surfaces or electrostatic deflection of single cells falling in droplets. The first method rarely achieves specificity because of nonspecific adhesion or variable detachment in the flowing medium, while the second method is extremely expensive, tedious, and difficult to keep sterile. A convenient and inexpensive separation method that maintains sterility and is performed in a nondrastic environment would be extremely useful in developing distinct cell populations. One promising method utilizes particles of magnetite coated with hydrophilic polymer carrying a cell-surface ligand (*1*). To be used generally, this separation requires a more convenient and controllable method of preparing the microspheres than has been demonstrated. The available method requires an intense source of gamma radiation, which is not often found in typical biological or chemical laboratories. On a commercial scale, such a preparation would be unwieldy. Microspheres much larger than the original magnetite particles (~ 50 nm) cannot easily be made this way, because the monomer is quickly exhausted in the formation of the predominant nonmagnetic product. This limitation on product particle size is significant because the very small coated magnetite particles form clumps in suspension, making selection from a distribution of cells impossible, while larger magnetic beads tend to stay separate. Further, separation of a small number of cells with the radiation-polymerized microspheres required 2 hours, possibly because a large nonmagnetic component was bound to the cells.

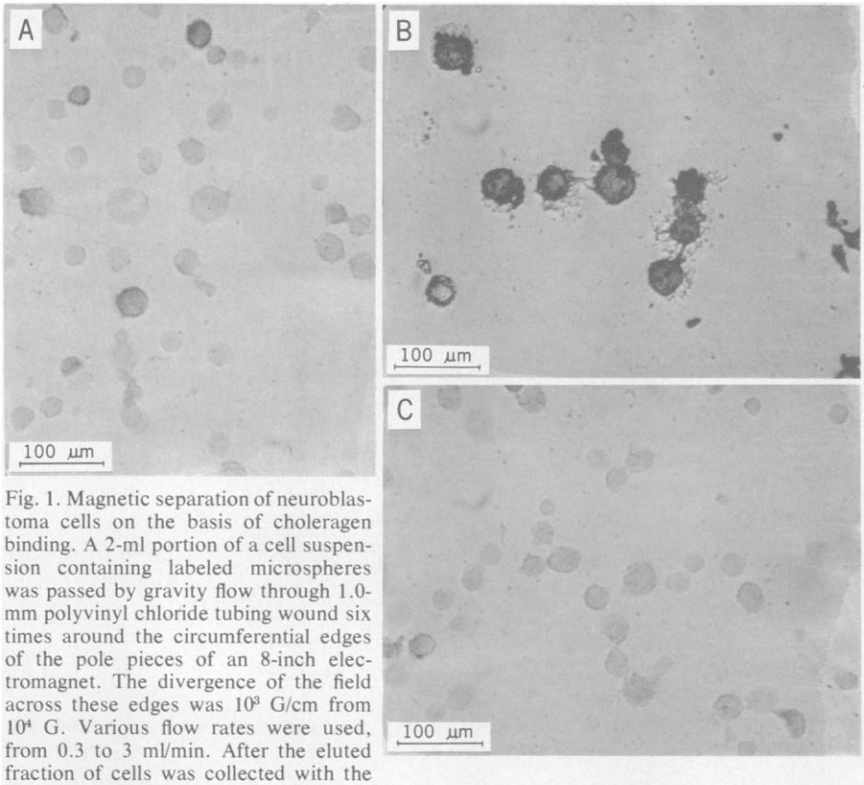

Fig. 1. Magnetic separation of neuroblastoma cells on the basis of choleragen binding. A 2-ml portion of a cell suspension containing labeled microspheres was passed by gravity flow through 1.0-mm polyvinyl chloride tubing wound six times around the circumferential edges of the pole pieces of an 8-inch electromagnet. The divergence of the field across these edges was 10^3 G/cm from 10^4 G. Various flow rates were used, from 0.3 to 3 ml/min. After the eluted fraction of cells was collected with the magnetic field on, the tubing was washed with medium into the eluted fraction. Then the magnetic field was turned off, and the retentate was washed through with more medium and collected separately as a single fraction. Separated and unseparated neuroblastoma cells were then grown in vitro for 24 hours and stained with HRP-choleragen, which had been prepared as described by Manuelidis and Manuelidis (*9*). Unfixed cells on cover slips were stained with HRP-choleragen (1 mg/ml) at 4°C for 1 hour. The cover slips were washed with PBS three times and fixed in Bouin solution. The method of Graham and Karnovsky (*10*) was used to develop the brown precipitate characteristic of dehydrogenated diaminobenzidine. The cover slips were then air-dried and mounted in Permount. (A) Neuroblastoma cells. (B) Cells binding microspheres and retained in the magnetic field (chloragen-positive cells). (C) Cells not binding microspheres and not retained in the magnetic field (choleragen-negative cells).

We have developed a facile method for preparing magnetic microspheres in high yield and controllable sizes and have used them to separate a minority population of neuroblastoma cells expressing the ganglioside G_{M1} on their surface membranes. Our system can be used in an ordinary laboratory to easily prepare magnetic microspheres which, when bound to specific ligands, enable rapid and facile cell separation.

The magnetic hydrogel microspheres can be prepared in 99 percent yield with predictable sizes if, instead of a conventional initiator system, the magnetite itself is used in a ferrous ion–persulfate redox polymerization system. The polymer forms exclusively around the magnetite particles, encapsulating them with beads of hydrogel. This reaction appears to be initiated by the ferrous ion diffusing from the particles and forming free radicals by reduction of the persulfate in the neighboring solution, since when the magnetite is omitted no polymerization occurs at room temperature (22°C).

In our example, 20 mg of allylamine and 20 mg of fluorescein isothiocyanate were shaken together in 10 ml of 0.01*M* borate buffer, *p*H 9.5, for 1 hour. This was then reacted at room temperature for 2 hours with 1.4 g of hydroxyethylmethacrylate, 0.2 g of *N*,*N*′-methylenebisacrylamide, 0.4 g of methacrylic acid, 0.05 g of sodium pyrophosphate, 0.03 g of ammonium persulfate, and 0.05 g of powdered magnetite (Wright Industries, Brooklyn, New York; 0.05-μm

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slurry), in 50 ml of H_2O at *p*H 3.9. The reactants were stirred under nitrogen in a round 100-ml flask with a paddle stirrer. The method of stirring was found to be critical: with the very vigorous stirring of an ultrasonic probe, less than 10 percent of the product was magnetic; with no stirring, the total yield was less than 10 percent. The product beads, 99 percent magnetic, were precipitated magnetically in a divergent 5000-G magnet, resuspended in 10 m*M* phosphate buffer at *p*H 7.0, and dialyzed against the same buffer. Diaminoheptane spacer groups were attached to the methacrylic acid groups by reacting 13.5 ml of magnetic beads (18 mg/ml, dry weight) in 10 m*M* phosphate buffer, *p*H 7.0, with 1.5 ml of diaminoheptane and 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, shaking with glass beads for 5 hours at 4°C. After dialysis against 0.1*M* NaCl followed by 10 m*M* phosphate buffer at *p*H 7.0, the product was activated by reacting in this buffer with 1.2 percent redistilled glutaraldehyde. The reaction mixture was shaken with 5-mm glass beads at room temperature for 1¼ hours, and then dialyzed against 10 m*M* phosphate buffer at *p*H 7.0. Choleragen (cholera enterotoxin; Schwarz/Mann, Orangeburg, New York) was attached to the activated spacer groups by reacting 50 mg of microspheres with 1 mg of toxin in 2 ml of 5 m*M* phosphate buffer at *p*H 7.0, shaking with glass beads at 4°C for 24 hours. We found by radioimmunoassay that in this reaction 50 percent of the choleragen bound to the microspheres, giving 1 mg of choleragen to 25 mg of microspheres. The coated beads were washed by centrifugation and resuspension three times into phosphate-buffered saline (PBS). The final suspension contained 1 mg (dry weight) of beads per milliliter of PBS.

We find the magnetic microspheres useful in separating cells from a line of neuroblastoma, some of which bear the ganglioside G_{M1}. The role of gangliosides in plasma membranes presents a complex biological problem (*2*). Ganglioside content has been correlated with properties of cell growth (*3, 4*), differentiation (*5, 6*), and transformation (*3, 4*). Sensitive methods for the detection of G_{M1} have been developed, utilizing the preferential affinity of choleragen for this molecule (*4, 6*). Variations in ganglioside content have been investigated in solid tumors, derived cell lines, and cell hybrids (*7*). The C-1300 neuroblastoma, a neuroectodermally derived transplantable tumor capable of expressing differentiated functions in vitro (*8*), has been shown to contain gangliosides. A clone of this tumor grown in vitro demonstrated individual heterogeneity in the cell-surface expression of G_{M1}, as indicated by the binding of either horseradish peroxidase (HRP)–conjugated choleragen (Fig. 1A), fluorescein-conjugated choleragen, or choleragen-conjugated fluorescent magnetic beads (not shown). The results of all three techniques show that 10 to 20 percent of the neuroblastoma cells bind choleragen. We have demonstrated specificity by the inhibition of the binding by excess G_{M1} in the cell medium.

Single-cell suspensions of this cloned line of C-1300 neuroblastoma cells grown to confluency in Dulbecco's modification of Eagle's basal medium (BME) (Grand Island Biological Co.) were used. From 2 to 5 × 10^6 cells were incubated with 0.1 to 1.0 mg (dry weight) of choleragen-conjugated magnetic microspheres at 4°C for 30 minutes. To remove excess unbound microspheres the cell suspension was layered on top of fetal calf serum and centrifuged at 800*g* for 10 minutes. This procedure was repeated twice. The cells were resuspended in 2 ml of Dulbecco's BME for the separation procedure. Our experiments with this clone demonstrate the specific binding of the coated magnetic beads, the separation of two populations of neuroblastoma cells according to their binding of choleragen-conjugated magnetic beads, and the partial characterization of the cells after separation.

The separations were completed in 1 to 6 minutes; the efficiency was not improved by the slower passage through the magnet. Magnetophoretic separation of the cells that bound the choleragen-conjugated beads was effected by using a divergent magnetic field, as described in the legend of Fig. 1, which shows the effectiveness of the cell separation. Unfractionated samples from two separations contained 12.5 and 15.0 percent choleragen-positive cells, whereas the retained and eluted fractions contained 99.0 and 99.5 percent and 1.0 and 1.5 percent, respectively. At least 200 cells were counted for each result.

Figure 2 shows the appearance of the beads bound to an individual cell and the distribution of constituents of beads and cell membrane over its surface, confirming our identification of these areas.

These results illustrate the magnetophoretic separation of cell populations distinguished solely on the basis of their cell-surface properties. The advantages of this technique over others, particularly fluorescence-activated cell sorting, are

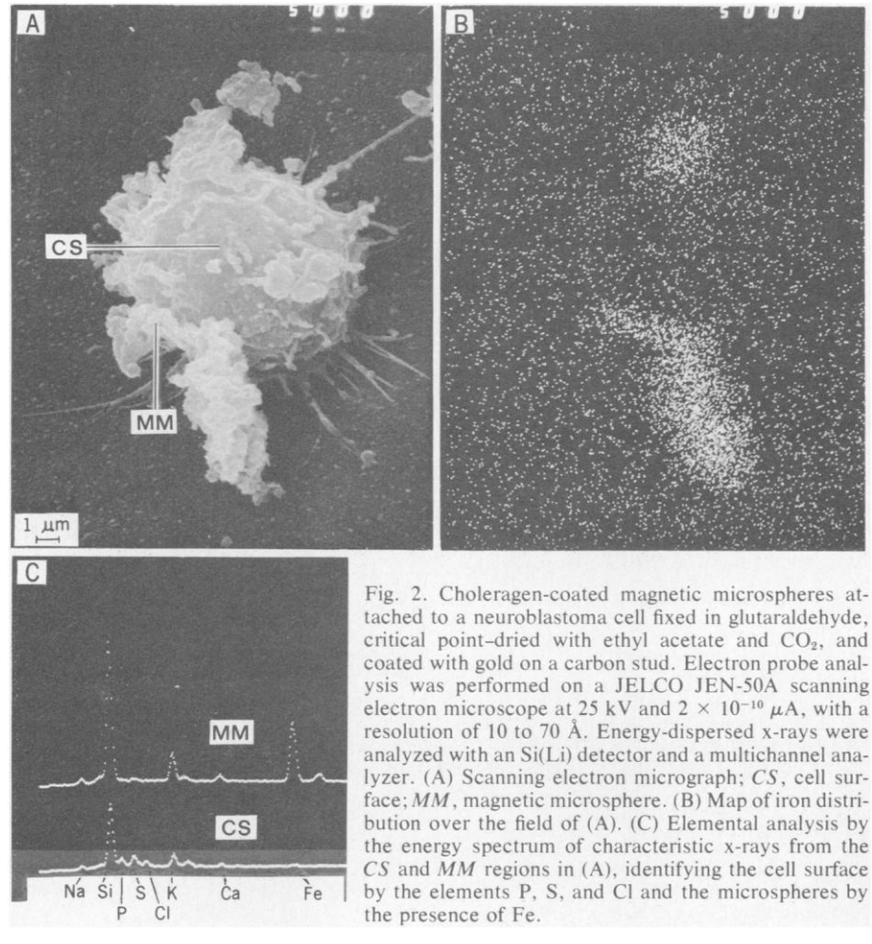

Fig. 2. Choleragen-coated magnetic microspheres attached to a neuroblastoma cell fixed in glutaraldehyde, critical point-dried with ethyl acetate and CO_2, and coated with gold on a carbon stud. Electron probe analysis was performed on a JELCO JEN-50A scanning electron microscope at 25 kV and 2 × 10^{-10} μA, with a resolution of 10 to 70 Å. Energy-dispersed x-rays were analyzed with an Si(Li) detector and a multichannel analyzer. (A) Scanning electron micrograph; *CS*, cell surface; *MM*, magnetic microsphere. (B) Map of iron distribution over the field of (A). (C) Elemental analysis by the energy spectrum of characteristic x-rays from the *CS* and *MM* regions in (A), identifying the cell surface by the elements P, S, and Cl and the microspheres by the presence of Fe.

that it is faster and easier to perform. We foresee no problems in using this technique for large numbers of cells.

The potential sensitivity of this separation technique is as good as or better than that of any other technique, since we can detect the presence of very small numbers of beads (not shown). In addition, we found that cells that contain such small numbers of beads can be readily separated on the basis of their differential magnetophoretic mobility. The separated cells were viable and grew again to confluency.

Plastic microspheres are widely used in a number of areas—for instance, as chromatographic fixed phases, as immunoprecipitants, for cell labeling, as supports for cell culture, and as vehicles for drug delivery. Addition of the magnetophoretic property to the particles could improve all of the associated techniques. The microspheres used in these various areas, however, differ widely in size. The method of preparation described here is, to the best of our knowledge, the only one capable of yielding sizes that cover this whole range, from tens of nanometers to tens of micrometers. With this development, expansion of the uses of the magnetic particles to these areas is now feasible.

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Reversible Cerebral Atrophy in Recently Abstinent Chronic Alcoholics Measured by Computed Tomography Scans

Abstract. *Eight chronic alcoholics received repeated computed tomography scans. Four, who maintained abstinence and functionally improved, showed partially reversible cerebral atrophy. Two nonabstinent patients and two abstinent patients who had completed functional improvement before the first scan showed no change in atrophy.*

Pathological (*1*) and pneumoencephalographic (*2*) data and studies using computed tomography (CT) scans (*3*), indicate that chronic alcoholism is associated with cerebral atrophy. Chronic alcoholism is also associated with a number of functional deficits in cerebral (*4*) and cerebellar function (*5*) which can show recovery when abstinence is maintained over periods of weeks to many months (*6*). Rapid recovery has been attributed to resolution of the alcohol withdrawal syndrome, which is considered a biochemical disorder (*7*). However, no mechanism has been proposed for the functional improvement observed weeks to months after the last drink. This gradual functional improvement suggested a structural rather than a purely biochemical basis for the recovery. Using the benign diagnostic technique of CT scanning (*8*), we noted a measurable decrease in the degree of cerebral atrophy in repeated CT scans in four of eight chronic alcoholics. We propose that this reversible atrophy represents a form of morphological plasticity in the central nervous system (CNS).

The CT scans were evaluated clinically by G.W. and subsequently evaluated by R.H., who was unaware of patients' clinical status. These evaluations were identical. In four cases, less atrophy, particulary cortical atrophy, was seen on the second scan. In another four cases no difference was seen between the first and second scans. Reversible atrophy was noted only in those patients who abstained from alcohol, showed clinical improvement, and had their initial CT scan before demonstrable clinical improvement was complete (Fig. 1).

The eight patients in this series are the first available for repeat clinical and CT

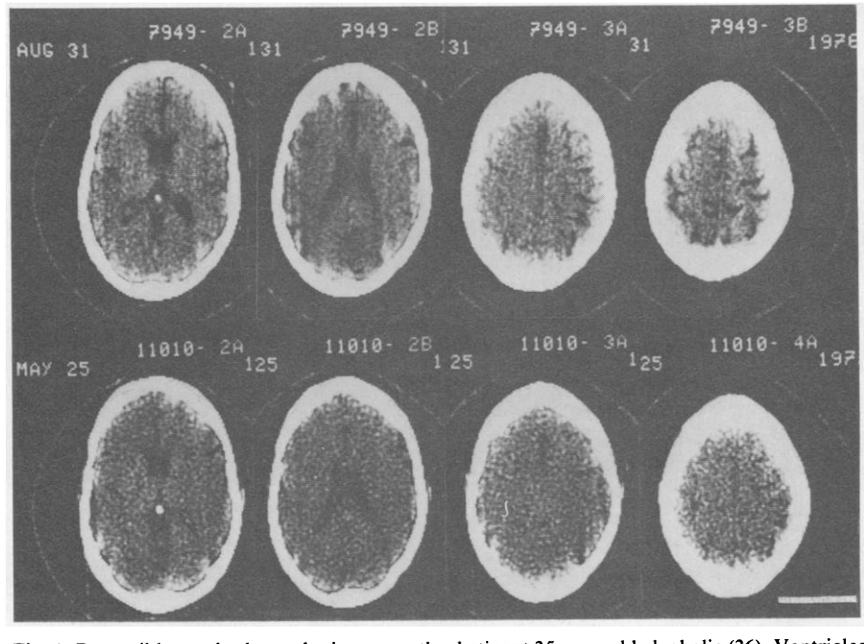

Fig. 1. Reversible cerebral atrophy in a recently abstinent 35-year-old alcoholic (*26*). Ventricles are dark areas in the central portion of pictures at left. The typical walnut-shell appearance of cortical sulcal atrophy is seen in pictures at right. The top row shows four contiguous CT scan cuts taken 4 weeks after the patient's last drink and demonstrates enlarged ventricles and numerous and enlarged cortical sulci. The bottom four CT scan cuts taken 8 months later show a reduction in ventricular size with a more marked reduction in size and number of visible cortical sulci. Variation in the photographic quality of the two scans cannot account for the observed changes. In a normal patient of the same age, ventricles are much smaller and sulci are not visible on CT scans. The calibration line represents 20 mm on standard Polariod photographs and 7.8 cm in actual tissue.

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