0273-2289/94/4546—0233$03.40

Cell Separations Using Targeted Monoclonal Antibodies Against Overproduced Surface Proteins

KALYAN R. TADIKONDA AND ROBERT H. DAVIS*

Department of Chemical Engineering, University of Colorado, Boulder, CO 80309-0424

ABSTRACT

The capacity of polystyrene microspheres with immobilized antibodies against type 1 pili of *E. coli* was measured. Using pure IgG-type monoclonal antibodies obtained by affinity purification, it was found that, at high concentrations of antibody in solution, 2.6 ± 0.4 mg of antibody immobilized/m² of surface area. The binding of piliating *E. coli* cells on incubation with antibody-coated microspheres at various microsphere-to-cell number ratios ranging from 1–3750 was studied. It was found that a maximum of 290 ± 50 cells were bound/microsphere, equivalent to a binding capacity of 2.9 ± 0.7 × 10^{10} cells/m² of surface area.

Index Entries: Cell separations; immobilized; monoclonal antibodies; cell adhesion; bacterial piliation.

INTRODUCTION

Separation of a particular functionally homogeneous subpopulation of cells from a mixture containing various different kinds of cells provides a challenge for scientists and researchers in biosciences, medicine, and biotechnology. Medical scientists encounter such challenges when mature-T cells need to be separated from bone marrow to prevent graft-vs-host disease in allogenic transplantations and to remove cancer cells from bone marrow for autologous transplantations. Isolation of rare cells types for cloning and biochemical studies is of interest to biologists, whereas

*Author to whom all correspondence and reprint requests should be addressed.

chemical engineers are interested in removal of nonproductive, segregant cells that usually grow faster than productive, plasmid-bearing cells in a recombinant bacterial fermentation.

Separation of cells can be achieved to various degrees by taking advantage of the variation in functional or physical properties between the different types of cells in a mixture. Physical properties such as size, charge, and density have been exploited in attempts to separate cells (*1*). Using these methods, however, a high degree of purification of a single population is difficult to achieve. Separation methods based on the ability of certain cells to differentially bind ligand molecules such as antigens, antibodies, receptors, proteins, lipids, and so on offer the greatest selectivity (*2*).

Nearly 99% of all cell separations to date are performed in centrifuges, usually as a part of research experimentation (*3*). Automated systems utilizing expensive instrumentation have been used for cell separation (*4,5*). Separations by sedimentation or centrifugal elutriation require a difference in size or density of the cell populations (*6–8*). Aqueous two-phase systems and electrophoresis are useful methods for cell separations (*9–12*) but need extensive study for each mixture from which desired cells need to be separated. Methods based on attachment of cells to affinity carriers usually offer the highest selectivity, because the ligands can be chosen to specifically bind the surface molecules on the desired or undesired cells. Antibodies and other affinity molecules that selectivity bind to a particular subpopulation of cells have been used to separate cells by panning (selective adhesion of desired or undesired cells) on Petri dishes coated with antibodies, or by using microspheres coated with antibodies (*13–19*). The microspheres with desired or undesired cells bound to their surfaces are then separated by differential sedimentation or magnetic separation, thus achieving effective cell separation (*16,17,19*). Columns containing gel or bead matrices with attached affinity molecules have been used to separate cells (*20*). The disadvantage of affinity gel columns is that they get clogged with cells and need periodic replacement. Microspheres can, however, be treated externally to remove bound cells, and then reused for cell separation.

Most of the research for cell separations to date has been with mixtures containing different kinds of mammalian cells, human cancer cells and bone marrow cells, T-cells and B-cells, or blood cells (*6,9,10,14,15,17, 20,21*). Partial separation of piliating cells of *E. coli* from nonpiliating cells of the same strain was achieved by selective adhesion of piliating cells to mannose-containing carriers—*Candida albicans* yeast cells (*22*). Successful separation of hyper-piliating *E. coli* cells was achieved from nonpiliating cells by exploitation of the flocculation properties of the former to separate them by differential sedimentation in an inclined settler. The strategy was used to maintain productive, plasmid-bearing, hyper-piliating cells in a bioreactor by selective recycle of the flocculated cells (*23*) and removal of nonpiliating, plasmid-free cells.

The goal in the present study is to develop a well-characterized system for cell separation in a high-cell density system, using targeted monoclonal antibodies for the removal of undesired cells from a suspension. Such a system will provide a good model for other cell separation problems using affinity ligands. The cells chosen here are two subpopulations of *E. coli* bacteria, one that piliates and one that does not. Monoclonal antibodies that bind to type 1 pili will be used to remove piliating cells from a suspension containing both piliating and nonpiliating cells. This paper describes studies aimed at immobilizing monoclonal antibodies that bind to type 1 pili by physical adsorption on polystyrene microspheres and characterizing the binding of piliating cells of *E. coli* to microspheres coated with monoclonal antibodies that bind to type 1 pili. The results can be used to estimate the amount of antibody-coated microspheres required to achieve effective separation of piliating cells from a suspension containing a mixture of piliating and nonpiliating cells, thereby enriching the suspension in nonpiliating cells.

MATERIALS AND METHODS

Production and Purification of Monoclonal Antibodies

Hybridoma cells that produce IgG type monoclonal antibodies (strain RT-1 245E1) that bind to type 1 pili were made by standard procedures at the University of Colorado Health Sciences Center in Denver (*19*). The hybridomas were grown in a continuous culture that was operated in a perfusion mode whereby viable cells were recycled into the bioreactor using an inclined settler that separates viable and nonviable cells based on the difference in their settling velocities (2.9 cm/h, and 1.1 cm/h, respectively) (*24*). The continuous culture was performed in a Celligen® bioreactor of 1.8 L working volume. Cells were grown in Dulbecco's Modified Eable's Medium supplemented with 5–10% fetal bovine serum. Dissolved oxygen was controlled at 50% of air saturation, and pH was controlled at 7.2. The bioreactor containing 1.6 L of medium was initially inoculated with 200 mL of a spinner flask culture containing 1.6×10^6 cells/mL. Cells were grown in a batch mode for 81 h, and the culture was then switched to continuous mode. The dilution rate was gradually increased from 0.5 to 1.7 d^{-1}. The culture was terminated after 500 h because of contamination. More than 25 L of culture product containing about 40 μg/mL of IgG antibodies were obtained. The culture supernatants were purified on a protein G affinity chromatography column to obtain the monoclonal antibodies in a pure form.

The culture product was pretreated to remove cell debris, and the concentrations of IgG in the supernatants so obtained were determined by

enzyme linked immunosorbent assays (ELISAs) (*25*). The antibodies from the supernatants were first precipitated by adding ammonium sulfate to 50% saturation at neutral pH. The precipitated antibodies were recovered by centrifugation at 1500*g* for 1 h at 4°C and redissolved in 1/10 original supernatant volume of phosphate buffered saline solution. The antibodies were then purified by protein G affinity chromatography in a high pressure liquid chromatography (HPLC) system. The adsorption buffer used was 0.1*M* sodium phosphate, pH 7.4, and the elution buffer used was 0.5*M* ammonium acetate, pH 3.0. The remaining antibody molecules in the column were stripped using 20% (v/v) acetic acid. The optical density of the eluting stream was continuously monitored by a UV detector at a wavelength of 280 nm. Fractions containing the antibody were pooled, and the pH was adjusted over ice to 7.2, using 2*M* tris solution. The antibodies were concentrated to 1/10 vol of the pooled fractions by ammonium sulfate precipitation. The final yield was 22.5 mg of pure antibody obtained from 2 L of culture supernatant.

IMMOBILIZATION OF MONOCLONAL ANTIBODIES ON POLYSTYRENE MICROSPHERES

The microspheres used in this study are polystyrene:2% divinyl benzene copolymer beads (Eastman Kodak Co., Rochester, NY, Cat #1358019). The diameter of the microspheres, as measured by a Coulter® Multisizer, ranges from 37–74 μm, with a volume-average diameter of 57.0 ± 0.2 μm at the 90% confidence intervals for nine measurements. The surface area available per gram of microspheres is 0.085 ± 0.003 m^2. Antibodies were physically adsorbed on the microspheres by the following procedure (*26*): A 2.5% (w/w) suspension of the microspheres was made in phosphate buffered saline solution containing 0.005% tween-20. The microspheres were washed three times with 0.1*M* borate buffer, pH 8.5. Since the antibodies were bound to the microspheres around this pH, the washing served as a preparative step to buffer the microspheres. Antibodies at known concentrations were added to microspheres pelleted in 1.6-mL microcentrifuge tubes from 0.5 to 1 mL of suspension, and the suspensions were incubated at room temperature by gentle stirring for 16 h after the tubes were filled up with borate buffer. The suspensions were centrifuged, and the supernatants stored separately. The remaining protein binding sites on microsphere surfaces were saturated by incubation for one-half hour in borate buffer containing 10 mg/mL bovine serum albumin. The microspheres were then washed three times in the same buffer and stored in PBS buffer contaning 10 mg/mL bovine serum albumin and 5% glycerol for determination of the antibody concentration on their surface by enzyme linked immunosorbent inhibition assays.

QUANTIFICATION OF ANTIBODIES ON MICROSPHERE SURFACES

Enzyme linked immunosorbent inhibition assays performed to determine antigen quantities on cell surfaces are described by Dodd and Eisenstein (27). These assays were used to quantify antibodies on microsphere surfaces. The procedures are as follows: 100 μL of PBS buffer containing 2.5 μg/mL antibodies that bind to type 1 pili were added to the wells of a micro-elisa plate. The plates were incubated for 16 h at 4°C. The plates were washed three times with PBS-0.05% Tween. The remaining protein-binding sites were blocked by adding PBS buffer containing 1 mg/mL gelatin to the wells and incubating for 4 h at room temperature. Serial twofold dilutions of antibodies starting from a concentration of 20 μg/mL and antibody-coated microspheres were then prepared, and 50 μL each were added to the wells. Some wells without addition of free antibody or microspheres served as controls. 100 μL of a goat-antimouse IgG-horseradish peroxidase conjugate diluted 1:4000 in PBS were added to the wells. Wells without the addition of any free antibody, microspheres or peroxidase served as blanks. After incubation for 4 h at room temperature, the wells were emptied, and the plates were washed once with PBS-Tween and two times in PBS. 100 μL of substrate solution consisting of *O*-phenylene diamine in 100 mL of citrate-phosphate buffer, pH 5.0, were added to the wells. After incubation for 10–30 min, the reaction was stopped using 25 μL of 12.5% sulfuric acid, and the results were read at 490 nm. Percent inhibition was calculated as follows:

$$\text{percent inhibition} = (OD_{\text{control}} - OD_{\text{well}} / OD_{\text{control}}) \times 100 \quad (1)$$

EXPERIMENTS WITH CELLS AND ANTIBODY-COATED MICROSPHERES

Wild-type piliating strain AT2475 (kindly provided by A. L. Taylor at the University of Colorado Health Sciences Center in Denver) was used in these studies. Cells were first grown in TTC medium (8 g Bacto® tryptone, 1 g Bacto® yeast extract, 5 g NaCl, 2 g KH_2PO_4, 50 mg 2,3,5-triphenyl tetrazolium chloride, and 10 g glucose/L of medium). When the cells were in exponential growth phase, 100 mL of suspension were centrifuged at 1500*g* to recover the cells. The cells were resuspended in PBS (pH 7.2–7.4) and plated on LB medium plates (10 g Bacto® tryptone, 5 g Bacto® yeast extract, 10 g NaCl, 0.2 g NaOH, and 15 g of Bacto® agar in 1 L of deionized water) after diluting appropriately in serial ten-fold dilutions so that the cell concentration in suspension could be determined. Experiments were performed as follows: In several 1.6-mL microcentrifuge tubes, 1 mL of

2.5% w/w suspension of antibody-coated microspheres was placed. Such a suspension had $2.13 \pm 0.07 \times 10^5$ microspheres (at the 90% confidence level for nine measurements). The microspheres were pelleted by centrifugation, and washed three times in sterile PBS solution. One milliliter of cell suspension at the desired concentration was added to the pellets obtained after the third wash, and then incubated by end-on-end rotation at room temperature. Each tube served as a sample. Samples were taken at known times, lightly centrifuged at 100*g* to differentially remove microspheres from suspension, and the supernatants were plated on LB plates after appropriate serial tenfold dilutions. The microspheres were then washed three times in sterile PBS solution to remove any unbound cells, and plated on LB plates to check for binding of piliating cells. Plates were incubated for 24 h, and only plates that had more than 30 colonies were counted. The number of cells bound to microspheres was determined by the difference in the number of cells in suspension before and after the suspension was incubated with microspheres. One set of experiments were also done for nonpiliating cell strain AT5050(pKLH7) (*28*).

RESULTS AND DISCUSSION

Results obtained for immobilization of monoclonal antibodies by enzyme linked immunosorbent inhibition assays are shown in Figs. 1–3. In Fig. 1, percent inhibition for free antibody in solution and for antibody-coated microspheres has been plotted against the logarithm of the dilution factor. Dilutions of antibodies in solution and microspheres that cause 50% inhibition were obtained by fitting a straight-line through the linear regions of the data. The amount of antibody causing 50% inhibition in both cases was considered the same, and, since the antibody concentrations in free solution were known, the amount of antibody on microsphere surfaces was calculated by multiplying with the appropriate dilution factors.

Figure 2 shows the amount of antibodies immobilized on microspheres as a function of initial concentration of antibody in solution. As can be seen in this figure, the amount of antibody immobilized per surface area reaches a peak value of 10.6 ± 0.7 mg/m^2 (at the 90% confidence level for three measurements) at an initial antibody concentration of 15 μg/mL, and thereafter at initial antibody concentrations of 50 μg/mL and over, reaches an average value of 2.6 ± 0.4 mg/m^2 (at the 90% confidence levels for twelve measurements). The variation in the amount of antibody immobilized with the initial antibody concentration in solution is thought to arise from differences in antibody orientation. Antibody orientation on anionic polyvinyl toluene microspheres at various pHs has been experimentally and theoretically estimated. The shape and dimensions of an IgG antibody molecule with its Fab arms stretched out to form a T-shaped molecule and the area occupied by the antibody molecule at various orientations are shown in Fig. 3 (*29*). The area occupied per IgG antibody

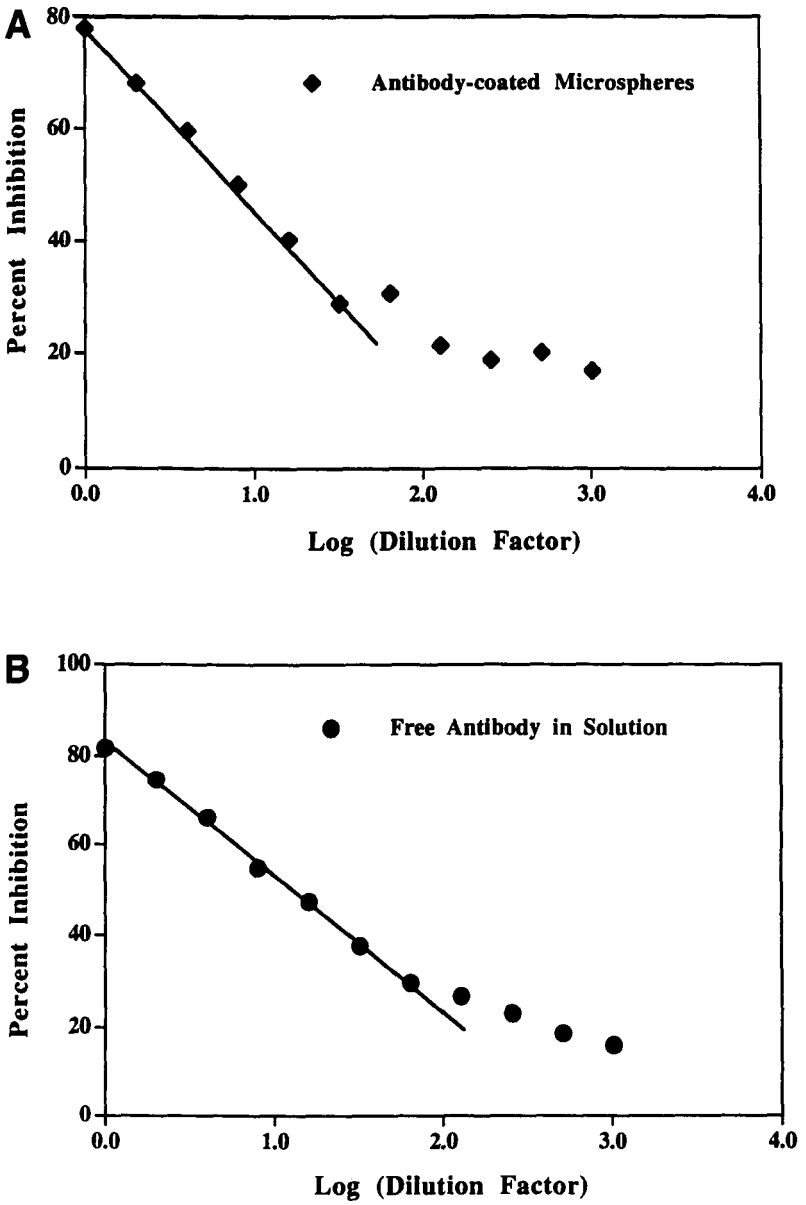

Fig. 1. Percent inhibition vs log of the dilution factor for determination of antibody concentration on microsphere surfaces.

molecule at initial antibody concentrations below 50 μg/mL peaks out at 2500 ± 200 Å^2 (with 90% confidence intervals), which is close to the estimated 3200 Å^2/molecule for the vertical orientations shown in Fig. 3. The area occupied per molecule at initial antibody concentrations above 50 μg/mL is at 10,000 ± 1000 Å^2 at the 90% confidence level, which is close to the estimated 10,700 Å^2/molecule for the horizontal orientation shown in Fig. 3. The differences in the estimated areas and the experimentally

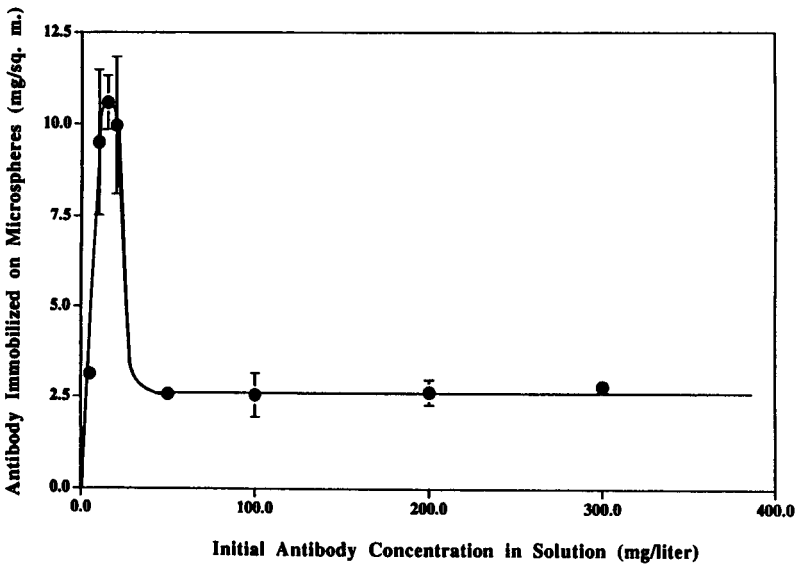

Fig. 2. Antibody immobilization on microsphere surface at various initial antibody concentrations in solution.

determined areas may be attributed to the fact that the antibody molecules could vary in size by virtue of the variable regions in both the Fc and the Fab regions, and also to the Fab arms not being quite as stretched as shown in Fig. 3. Similar trends were reported previously (*30*), although the amount of antibody immobilized per surface area at initial antibody concentrations lower than 50 μg/mL was not as high as observed by us. As proposed earlier (*30*), antibody conformation at initial antibody concentrations greater than 50 μg/mL that cause surface crowding is favored to be in the horizontal orientation with larger area. The antibody immobilization values at initial concentrations greater than 50 μg/mL suggest a binding of 9.8×10^{15} molecules/m^2 of surface area on microspheres, based on the antibody molecular weight of 160,000 daltons. The horizontal orientation occurring at high-concentration incubation was chosen for experiments to bind piliating cells because, in this case, both of the antigen-binding sites are available for cell binding, in contrast to the vertical orientation at low-concentration incubations for which only one antigen-binding site is available for cell binding.

Experiments in which antibody-coated microspheres were incubated with piliating cells or with nonpiliating cells were performed to determine the maximum number of piliating cells that would bind per surface area as well as to check for any nonspecific binding. Figure 4 shows the cell number in piliating and nonpiliating cell suspensions as functions of time of incubation with microspheres (here, the cell number equals the cell

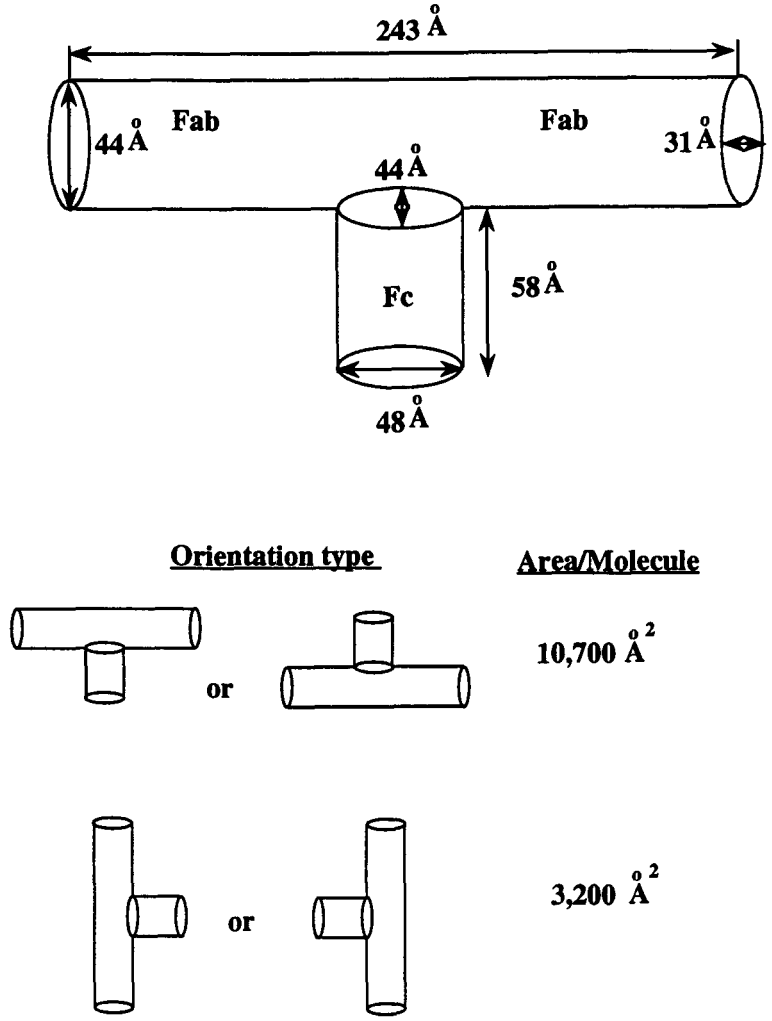

Fig. 3. IgG antibody molecule: shape, dimensions, and orientations on microsphere surfaces (adapted from Bagchi and Birnbaum, 1981 [29]).

concentration since the volume of suspension was 1 mL). As can be seen in this figure, the nonpiliating cell number remains unchanged, suggesting that the nonpiliating cells do not bind to antibody-coated microspheres. No colonies were observed on plates on which microspheres were plated subsequent to their incubation with cells. For the experiment with piliating cells, however, the piliating cell number continually decreases until the seventh hour. Colonies were observed on plates on which microspheres were plated after incubation and three washes in PBS. The results obtained from the experiments described above confirm the specificity of binding of piliating cells to the microspheres.

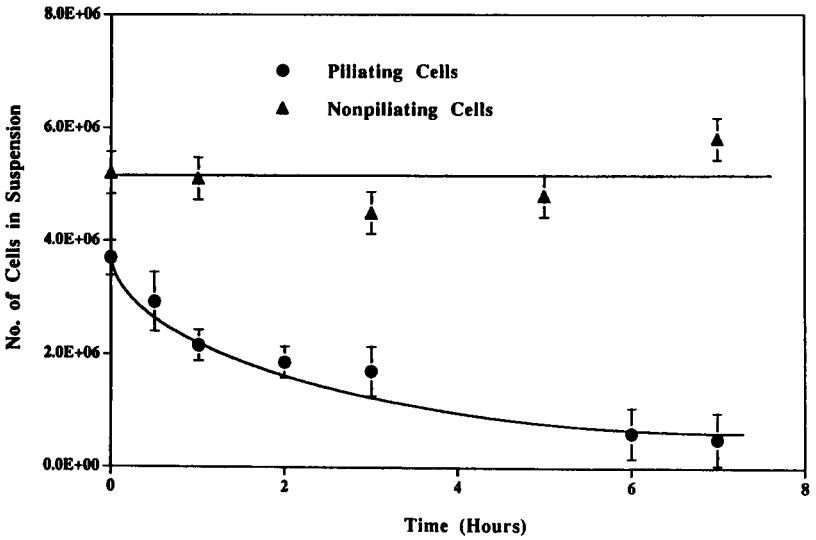

Fig. 4. Cell numbers in suspension as a function of time of incubation in experiments for binding of piliating and nonpiliating cells to antibody-coated microspheres.

The experiments with piliating cells were repeated for various concentrations of piliating cells in suspension (or various ratios of piliating cells to microspheres) and increasing number of cells bound to the same number of microspheres with increasing concentration. The results plotted with the number of cells bound per microsphere as a function of number cells incubated per microsphere after 3 h of incubation are shown in Fig. 5 (in most experiments no significant further binding of piliating cells to antibody-coated microspheres was observed after the third h of incubation). The large error bars in this graph can be explained by the fact that the values obtained are differences in the number of cells in the supernatant before and after incubation, and the errors propagate in such a calculation. The results obtained from these experiments suggest that a maximum average of 290 ± 50 cells bind per microscope (at the 90% confidence level for twelve measurements). This is equivalent to a binding of $2.9 \pm 0.7 \times 10^{10}$ cells/m^2 of surface area. Thus, 34 μm^2 of surface area is required for the binding of each cell, on average. This is an order-of-magnitude larger than the area of a single *E. coli* cell footprint (3 μm × 1 μm dimensions). Since 1.0 m^2 surface area has 9.8×10^{15} antibody molecules, 3.4×10^5 molecules bind each cell, on average. The insight obtained here is vital for estimation of antibody amounts and microsphere surface area required for selective removal of piliating cells from a mixture containing both piliating and nonpiliating cells.

Our long term goals are to develop strategies for recovery of bound piliating cells from microsphere surfaces, and to then use antibody-coated microspheres for selective removal of piliating cells from a mixture containing both piliating and nonpiliating cells, thereby enriching the

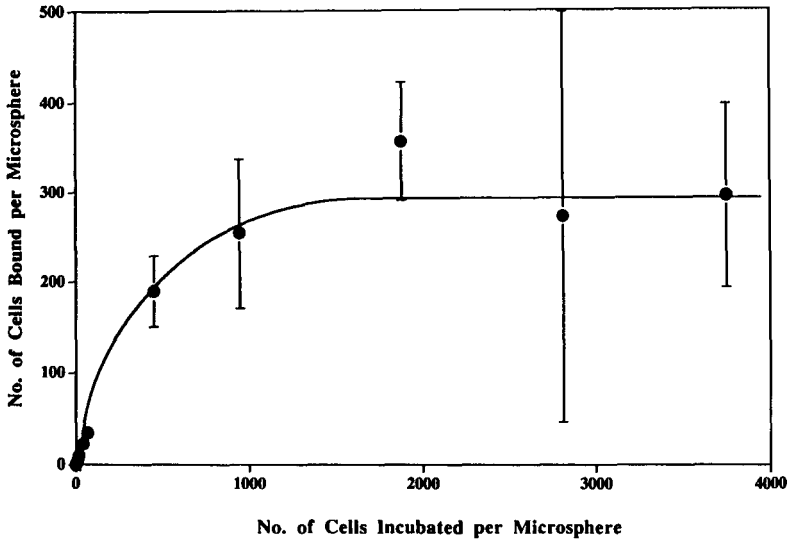

Fig. 5. Number of piliating cells bound per microsphere as a function of number of cells incubated per microsphere after 3 h of incubation.

suspension in nonpiliating cells. The strategies developed for bound-cell removal will then be used to recover piliating cells as well as the antibody-coated microspheres. Such a study will provide a thorough characterization of cell separations using targeted monoclonal antibodies from a high-cell density suspension.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant No. BCS-8912259.

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