

Hollow-Fiber Assay for Ligand-Mediated Cell Adhesion

Robert E. Nordon,* Alan Shu, Fernando Camacho, and Bruce K. Milthorpe

Graduate School of Biomedical Engineering, University of New South Wales, Sydney, Australia

Received 22 April 2003; Revision Received 8 August 2003; Accepted 8 August 2003

Background: The investigation of receptor-ligand interactions in the cellular context presents significant technical challenges, first, to immobilize the ligand in a manner that preserves functional properties and, second, to relate ligand properties to cell adhesion and other cellular processes.

Methods: Ligand-mediated cell adhesion was characterized by the development of a cellulose hollow-fiber adhesion assay in which ligand (protein A) was immobilized onto the cellulose membrane as a recombinant fusion protein containing a cellulose-binding domain affinity tag. Modules containing single cellulose hollow fibers were connected to a micro-flow system for cell deposition and detachment with fluid shear stress. The cell adhesion process that occurred inside a segment of hollow fiber was observed in real time by using an inverted microscope equipped with a CCD camera and digital frame grabber.

Image analysis software was developed to count cells and record digital images.

Results: Cell adhesion strength was characterized by counting the number of cells that were detached by application of fluid shear stress with values that ranged from 2.3 to 185 dyne/cm². The median shear stress of detachment of KG1a cells was directly related to the duration of membrane contact and the amount of immobilized monoclonal antibody (anti-CD34).

Conclusions: The hollow-fiber assay provides a general method to determine functional properties of molecular domains that interact with cell surface receptors and markers. Cytometry Part A 57A:39–44, 2004.

© 2003 Wiley-Liss, Inc.

Key terms: cell adhesion; recombinant fusion proteins; cellulose-binding domain; cellulose hollow fibers

Receptor-mediated cell adhesion required for cell-to-cell and cell-to-matrix interaction is a ubiquitous process (1–3). Experimental investigation has focused on relating biophysical and biochemical properties of cell and matrix components to the strength of cell attachment (4–7). Development of methods for presentation of binding domains for the study of cell adhesion poses a significant technical challenge, first, to immobilize the ligand in a manner that preserves functional properties and, second, to relate ligand properties to cell adhesion.

Cell adhesion strength is measured by counting the number of cells that remain attached to a solid substrate after application of a well-defined detachment force such as fluid shear stress. Flow cells that have parallel plate or tubular geometry generate uniform fluid shear stress at the attachment surface, and cells are subjected to the same detachment force (3,7,8). In previous studies, the process of ligand-mediated cell attachment was investigated by chemical immobilization of antibodies onto a glycophasic glass plate that formed the base of a parallel-plate flow cell (9). Here we present an alternate method for measuring ligand-mediated cell adhesion. Instead of modifying substrate chemistry, ligand was immobilized with a bifunctional fusion protein containing an affinity tag for the solid substrate.

This method of ligand presentation was investigated by development of a cellulose hollow-fiber adhesion assay in

which a recombinant cellulose-binding domain (CBD) isolated from *Clostridium celovorans* was used as the affinity tag. Monoclonal antibodies for cell capture were immobilized onto hollow fibers by using staphylococcal protein A (ProtA) as the CBD fusion partner (10).

MATERIALS AND METHODS

Apparatus for Measurement of Ligand-Mediated Cell Adhesion

Figure 1 depicts the apparatus for measurement of ligand-mediated cell adhesion. Flow was generated with a syringe pump (B. Braun-Melsungen) using discrete gear ratios and flow rates. The position of the three-way tap determined whether the flow system was connected to a 100- μ l syringe, which was operated manually, or a 5-ml syringe pump. The hollow-fiber module consisted of a Cuprophan (regenerated cellulose; Membrana GmbH, Wuppertal, Germany) dialyzer fiber with an external diameter of 200 μ m and a wall thickness of 7 μ m mounted

Contract grant sponsor: Australian Research Council; Contract grant sponsor: Becton Dickinson Immunocytometry Systems.

*Correspondence to: Dr. Robert Nordon, Graduate School of Biomedical Engineering, University of New South Wales, Sydney 2052, Australia. E-mail: r.nordon@unsw.edu.au

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.a.10091

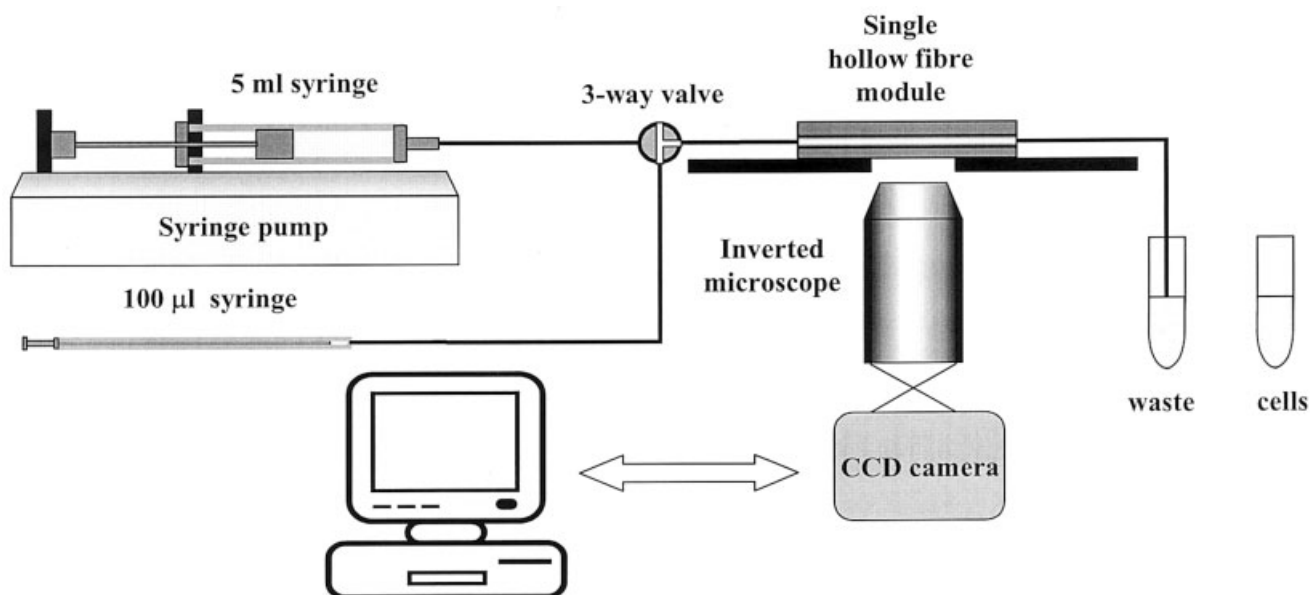


FIG. 1. Schematic of apparatus for measurement of ligand-mediated cell adhesion. Flow (11–870 $\mu\text{l}/\text{min}$) was generated by a 5-ml syringe pump connected to the inlet port of a hollow-fiber module. Cells were drawn into the module with the 100- μl syringe. Cell adhesion within a segment of fiber was viewed and analyzed in real time by using an inverted microscope equipped with a CCD camera, image-capture board, and PC-based software (WIT).

at the bottom of a 35-mm polystyrene tissue culture dish (Iwaki Glass, Tokyo, Japan) with inlet and outlet ports for filling the lumen of the hollow fiber with cells and buffer. The hollow-fiber module was mounted on an inverted microscope (Olympus IX70, Mount Waverely, Victoria, Australia) equipped with a CCD camera and image-capture board (Pulnix TM1001, Total Turnkey Solutions Pty. Ltd., Coburg, Victoria, Australia). It was necessary to hydrate and immerse the Cuprophane fibers in buffer so that cells that had deposited inside fibers could be visualized by light microscopy. WIT image analysis software (Total Turnkey Solutions Pty. Ltd.) was used to count the number of deposited cells and to store images in TIF format.

The mobile fluid phase was Dulbecco's phosphate buffered saline (PBS) containing 5 mg/ml of bovine serum albumin (BSA). The module was centered over the microscope objective (10 \times) so that the same segment of hollow fiber (2 mm length) could be microscopically analyzed in real time during cell deposition and detachment phases of the experiment. The three-way tap was rotated into the position that connected the 100- μl syringe to the hollow-fiber module, and a cell suspension (5 million cells/ml) was drawn into the hollow fiber module. This resulted in deposition of approximately 150 cells per millimeter length of hollow fiber. After cells had settled inside the fiber, an image was captured, and the number of cells was counted for a 2-mm segment of fiber. After a period of deposition (4 to 8 min), the syringe pump was connected to the fiber module by altering the position of the three-way valve. The syringe pump was started, and after cell detachment was complete, typically 60 s, the remaining number of attached cells was counted. The fraction of cells that was bound in the fiber segment was calculated:

fraction bound after shear

$$= \frac{\text{number of cells remaining bound after shear}}{\text{number of cells deposited}}$$

After the experiment, the fiber module was flushed with buffer and could be reused up to four times without significant degradation of the affinity membrane.

The fluid shear stress was calculated with the assumption of a well-established Poiseuille flow in a tube (11). The calculated fluid leak across the semipermeable Cuprophane membrane that results from an axial pressure gradient during flow was negligible (Cuprophane membrane permeability $\sim 1.5^{-12}$ cm) (12,13).

$$\tau = \frac{4Q\mu}{\pi r^3}$$

where τ is the wall shear stress, Q is the flow rate, μ is the viscosity, and r is the radius of the fiber ($\mu = 0.01$ poise and $r = 0.01$ cm). Flow rates of 11 to 870 $\mu\text{l}/\text{min}$ generated shear stresses of 2.3 to 185 dynes/cm².

Construction of Single Hollow-Fiber Modules

A single Cuprophane hollow fiber was mounted at the bottom of a 35-mm polystyrene tissue culture dish (Iwaki Glass) connected to inlet and outlet ports for deposition of cells inside the fiber and by applying fluid flows and shear stress for cell detachment (Fig. 2). Dr. Ulrich Baurmeister (Membrana GmbH) kindly provided Cuprophane (regenerated cellulose) dialyzer fibers. Injection ports were made from 10-mm lengths of 23-gauge stainless steel tubing (outer diameter, 0.8 mm) cut from 23-gauge hypodermic

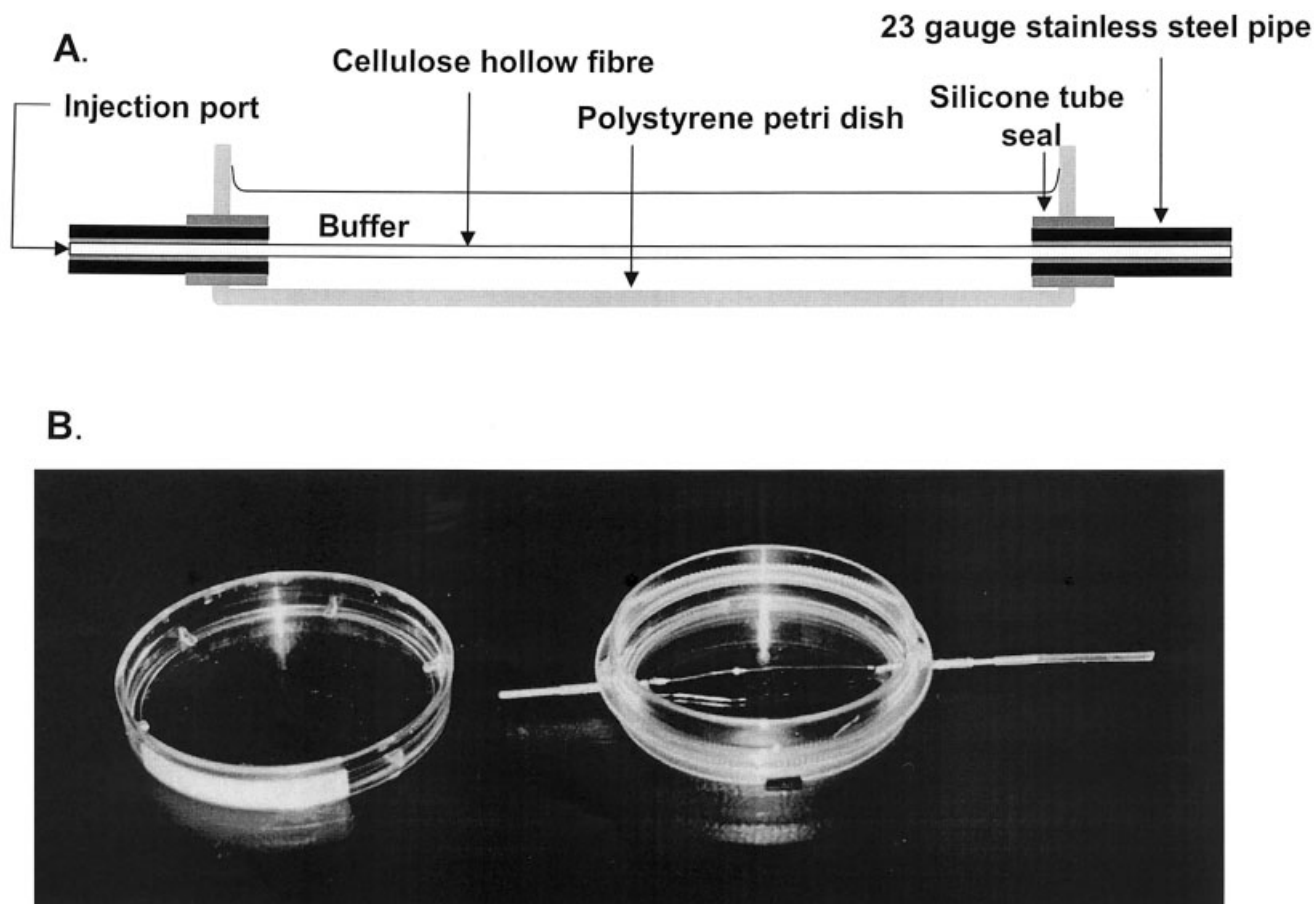


FIG. 2. Single hollow-fiber module. **A:** Schematic diagram of a section through a cellulose hollow-fiber module. Single cellulose hollow fibers were mounted at the base of a 35-mm polystyrene tissue culture dish and connected to stainless steel tube injection ports (23 gauge) that passed through holes in the wall of the culture dish. The cellulose hollow fiber was potted in silicone elastomer inside the stainless steel injection ports. The tissue culture dish was filled with buffer solution before cells were injected into single fibers via injection ports. **B:** Photograph of hollow-fiber module.

needles, which were cemented into 0.9-mm holes cut into the wall of the Petri dish. Silastic tubing (0.51 mm inner diameter \times 0.94 mm outer diameter) cut into 5-mm segments (catalog no. 508-002, Dow Corning, APS Chemicals, Sydney, Australia) was passed through the holes before insertion of stainless steel tubes to create a seal between the Petri dish and the outside of the stainless steel tube. Epoxy cement was used on the outside of the dish to increase the rigidity of the joint. A single Cuprophane hollow fiber was threaded through the inlet port into the Petri dish and out through the outlet port while taking care not to crush the segment of fiber that was inside the module. The joint between the hollow fiber, stainless steel tube, and inside wall of the Petri dish was sealed with silicone elastomer (Sylgard 184, Dow Corning Pty. Ltd., Sydney, Australia), which was thickened by partial curing at 37°C before application to the joint. The ends of the Cuprophane fiber were cut flush with the outlet and inlet of the injection ports with a razor blade. The module was connected to the flow system (Fig. 1) with silastic tubing (0.51 mm inner diameter \times 0.94 mm outer diameter). The

Petri dish was filled with 2 ml of buffer so that cells inside the fiber could be visualized with an inverted microscope.

Immobilization of Ligands

The recombinant protein, ProtA fused to CBD (ProtA-CBD) was purchased from Sigma-Aldrich (Sydney, Australia). Hollow-fiber modules were injected with a 40- μ g/ml solution of ProtA-CBD in PBS and incubated overnight at room temperature. For some experiments the immunoglobulin G2a monoclonal antibody (mAb) to the CD34 antigen (MHCD3400, Caltag Laboratories, Edward Keller, Sydney, Australia) was bound to fibers after overnight adsorption of ProtA-CBD. Coated fibers were incubated for 2 h at room temperature with a 1:10 or 1:20 dilution of the purified antibody (200 μ g/ml).

Culture and Labeling of KG1a Cells

The acute myelogenous leukemia cell line KG1a, which expresses the CD34 antigen, was grown in suspension culture (RPMI-1640 medium supplemented with 10% fetal bovine serum) (14). Cells were harvested during the mid-

log growth phase and washed with PBS supplemented with 5 mg/ml of BSA. KG1a cells were bound directly to Cuprophane membranes that were coated with anti-CD34 mAb (see above). For indirect capture by the ProtA immunoadsorbent, KG1a cells suspended in PBS with 5 mg/ml of BSA were incubated with 10 μ l of mAb (MHCD3400) per million cells for 30 min at 4°C, followed by washing and suspension in PBS with 5 mg/ml of BSA.

Statistical Methods

Unless stated otherwise, replicate data are shown as the mean \pm standard error of the mean for quadruplicate experiments. Means were compared with the unpaired T test. The logistic function was fitted to (x,y) data pairs expressing the relationship between shear stress of detachment and the fraction of cells that were still attached:

$$y = \frac{ax^b}{1 + ax^b}$$

where x is the detachment shear stress and y is the fraction of cells that remain bound. The coefficients a and b were estimated by nonlinear regression analysis with minimization of the squared residual (Systat software).

The median shear stress of detachment is the shear stress required to detach 50% of the total number of deposited cells ($y = 1/2$) and was calculated with the estimated coefficients of the "best-fit" logistic equation:

$$\text{median shear stress of detachment} = a^{-1/b}$$

RESULTS

Initially we investigated whether interactions between the cell surface receptor, CD34, and the mAb (MHCD3400) or the Fc region of the mAb and ProtA could mediate cell adhesion. Figure 3 shows that KG1a cells could be bound directly or indirectly to the cellulose membrane and that binding was mediated by the interaction of ProtA and anti-CD34 mAb. For this series of experiments, the cell deposition time was 5 min and the shear stress of detachment was 5 dyne/cm².

Only $5 \pm 2\%$ ($n = 4$) of unlabeled KG1a cells bound to ProtA-CBD-coated fibers (Fig. 3, bar D), demonstrating a relatively low level of nonspecific interaction of KG1a with immobilized ProtA-CBD ($P < 0.001$). Unmodified cellulose fiber did not bind KG1a cells (data not shown). Direct binding of KG1a cells to cellulose membrane was possible with hollow-fiber modules that were coated with ProtA-CBD, followed by incubation with anti-CD34 mAb at a 1:10 dilution in PBS for 2 h at room temperature (Fig. 3, bar C: $93 \pm 5\%$). When KG1a cells were incubated with antibody, $87 \pm 9\%$ of cells were bound to cellulose hollow fibers coated with ProtA-CBD (Fig. 3, bar B). Incubation of fiber and cell with antibody did not block cell attachment (Fig. 3, bar A: $88 \pm 2\%$), suggesting that not all ProtA binding sites were blocked by mAb.

We next investigated the strength of attachment by determining the number of cells that remained bound

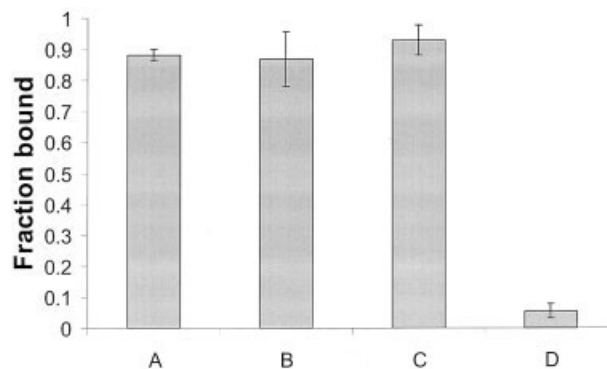


FIG. 3. Direct and indirect attachment of KG1a cells to hollow fibers coated with ProtA-CBD. KG1a cells were injected into hollow fibers followed by 5 min of deposition (no flow). The bar graph shows the fraction of KG1a cells that remained bound after they were subjected to a detachment force of 5 dyne/cm² ($n = 4$, mean \pm standard error of the mean). Cellulose hollow-fiber modules were incubated overnight with ProtA-CBD (40 μ g/ml). **A:** The hollow fiber and KG1a cells were incubated with anti-CD34 mAb. **B:** KG1a cells were incubated with anti-CD34 mAb. **C:** Hollow fiber was incubated with anti-CD34 mAb. **D:** Unlabeled KG1a cells served as negative control experiments.

with differing detachment shears. At higher shear stress (50 dyne/cm²), attached cells elongated (Fig. 4) and formed new attachments with the cellulose substrate. The cell footprint did not reverse shape when flow was stopped. We suspected that rapid formation of receptor-ligand complexes resulted in multipoint cell attachment that was strong enough to irreversibly deform the cytoskeleton.

The rate of cell attachment was investigated further by changing deposition time (4 vs. 8 min) and detachment shear stress (2.3–185 dyne/cm²). A detachment curve for each deposition time was generated by plotting detachment shear stress on the x axis versus the fraction of cells that remained bound on the y axis (Fig. 5). Hollow fibers were incubated overnight with ProtA-CBD (40 μ g/ml). KG1a cells that were labeled with anti-CD34 mAb were injected into hollow fibers, followed by 4 (squares) or 8 (triangles) min of deposition (flow stopped). The logistic equation (equation 3) was fitted to the data. The calculated median shear stress of detachment (equation 4) doubled over this period (26.8 vs. 47.6 dyne/cm²).

The strength of KG1a attachment was then related to the amount of immobilized mAb. Hollow fibers were incubated overnight with ProtA-CBD, followed by incubation with anti-CD34 mAb for 2 h at room temperature. Two different Cuprophane membrane-coating densities of mAb were generated by dilution of the incubating antibody (1:10 and 1:20).

Figure 6 shows detachment curves for the 1:10 (squares) and 1:20 (circles) antibody dilutions. Data points and error bars represent the mean and standard error of the mean for triplicate experimental runs. Equation 3 was fitted to the data to estimate the coefficients a and b . Equation 4 was used to estimate the median shear stress of detachment. The median shear stresses of detachment for

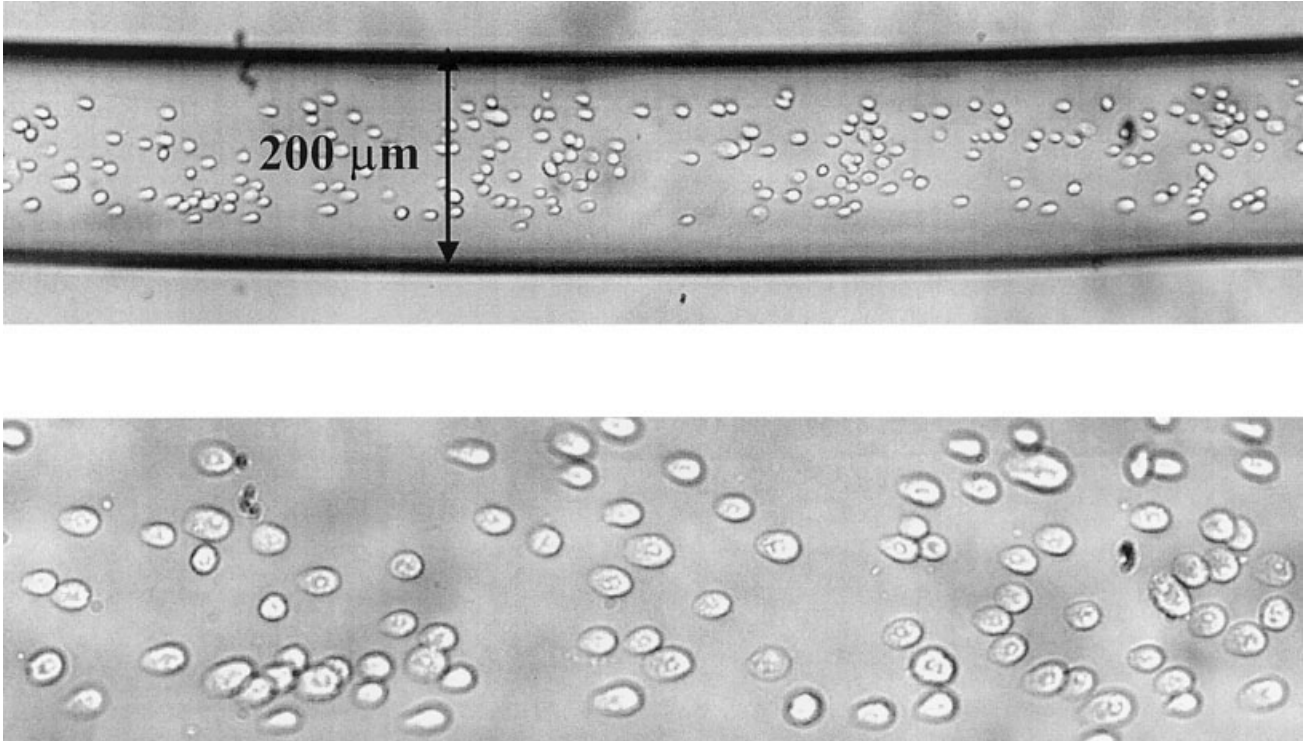


FIG. 4. Attached cells elongate at high detachment shear stress. Cellulose hollow-fiber modules were incubated overnight with ProtA-CBD (40 $\mu\text{g}/\text{ml}$). KG1a cells were incubated with anti-CD34 mAb and injected into hollow fibers, followed by 5 min of deposition (no flow). Bound KG1a cells elongated after they were subjected to a detachment force of 50 dyne/cm^2 .

1:20 and 1:10 antibody dilutions were 10 dyne/cm^2 and 17.4 dyne/cm^2 , respectively.

DISCUSSION

Ligand-mediated cell adhesion is characterized by the formation of receptor-ligand bonds in a time-dependent

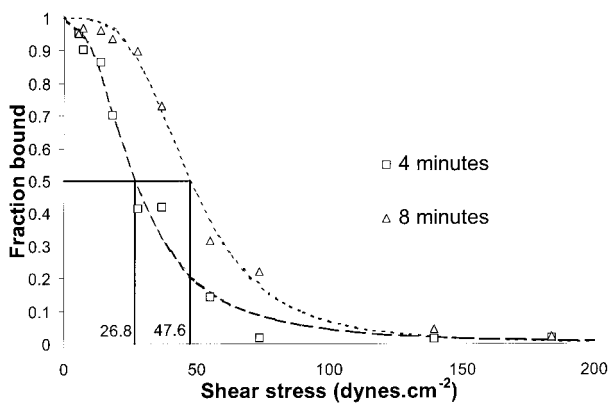


FIG. 5. Effect of cell deposition time on cell adhesion. Hollow fibers were incubated overnight with ProtA-CBD (40 $\mu\text{g}/\text{ml}$). KG1a cells labeled with anti-CD34 mAb were injected into hollow fibers followed by 4 (squares) or 8 (triangles) min of deposition at zero flow. Each data point represents a single run. The fraction of cells that remained bound (vertical axis) was inversely related to the detachment shear stress (horizontal axis). The median shear stresses of detachment for 4 and 8 min of cell deposition were 26.8 and 47.6 dyne/cm^2 , respectively.

fashion with changes in cytoskeletal organization and cell shape (4,5). The stochastic nature of this complex interaction has been characterized by determining adhesion strength population distributions at different time inter-

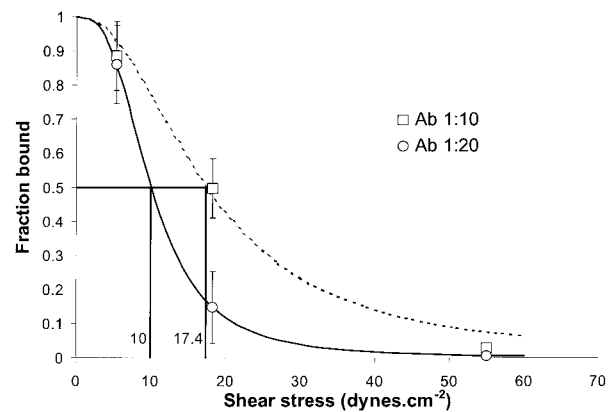


FIG. 6. Effect of immobilized mAb on cell adhesion. Hollow fibers were incubated overnight with ProtA-CBD (40 $\mu\text{g}/\text{ml}$) followed by incubation with anti-CD34 mAb for 2 h at room temperature. The monoclonal antibody stock ($\sim 200 \mu\text{g}/\text{ml}$) was diluted 1:10 (squares) or 1:20 (circles). Data points and error bars represent the mean and standard error of the mean for triplicate experiments. The vertical axis shows the fraction of cells that remained bound after they were subjected to a detachment shear stress (horizontal axis). The median shear stresses of detachment were 10 and 17.4 dyne/cm^2 for 1:20 and 1:10 antibody dilutions, respectively.

vals after cell deposition (9). The hollow-fiber adhesion assay is a direct method to determine the relation between fluid shear stress, cell detachment, and ligand properties (Figs. 5 and 6) and provides another method to study the biophysics of cell adhesion. The technique has the advantage that cells can be visualized during the attachment and detachment process, which may be useful for studying the organization of cytoskeletal elements and receptors under static or stressed hydrodynamic conditions (Fig. 4). The technique would be particularly powerful in combination with vital fluorescent staining of cytoskeletal elements and confocal microscopy.

In the past, ligand has been immobilized onto substrates by physical adsorption or covalent chemistry. Instead of modifying the chemistry of the substrate to immobilize ligand, recombinant DNA techniques were used to create fusion proteins that link a substrate affinity tag (CBD) to a ligand domain (ProtA). Presumably, the affinity tag interacts with the substrate in a steric fashion so that the ligand domain is oriented away from the surface, resulting in high binding capacity and low levels of nonspecific interaction.

The primary motivation for development of the single-fiber assay was to characterize the functional activity of novel CBD chimeras that display cell adhesion or trophic domains (data not shown). For example, Doheny and coworkers (15) synthesized a CBD fusion protein with stem cell factor (SCF) that interacts with the c-kit cell surface membrane receptor. The SCF-CBD chimera effectively immobilized and displayed SCF onto bacterial crystalline cellulose particles. They demonstrated that cellulose particles coated with SCF are colocalized with the receptor for SCF (c-kit) on B6SutA cells. Subsequent work will examine the potential role for immobilized ligands using hollow-fiber-based systems for cell selection and expansion (16,17).

LITERATURE CITED

1. Skubitz AP. Adhesion molecules. *Cancer Treat Res* 2002;107:305-329.
2. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;110:673-687.
3. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991;65:859-873.
4. Cozens-Roberts C, Lauffenburger DA, Quinn JA. Receptor-mediated cell attachment and detachment kinetics. I. Probabilistic model and analysis. *Biophys J* 1990;58:841-856.
5. Cozens-Roberts C, Quinn JA, Lauffenburger DA. Receptor-mediated cell attachment and detachment kinetics. II. Experimental model studies with the radial-flow detachment assay. *Biophys J* 1990;58:857-872.
6. Zhu C, Long M, Chesla SE, Bongrand P. Measuring receptor/ligand interaction at the single-bond level: experimental and interpretative issues. *Ann Biomed Eng* 2002;30:305-314.
7. Cozens-Roberts C, Quinn JA, Lauffenburger DA. Receptor-mediated adhesion phenomena. Model studies with the radial-flow detachment assay. *Biophys J* 1990;58:107-125.
8. Abbitt KB, Nash GB. Characteristics of leucocyte adhesion directly observed in flowing whole blood in vitro. *Br J Haematol* 2001;112:55-63. [Erratum in *Br J Haematol* 2001;113:844.]
9. Nordon RE, Milthorpe BK, Schindhelm K, Slowiaczek PR. An experimental model of affinity cell separation. *Cytometry* 1994;16:25-33.
10. Shpigel E, Goldlust A, Eshel A, et al. Expression, purification and applications of staphylococcal protein A fused to cellulose-binding domain. *Biotechnol Appl Biochem* 2000;31(pt 3):197-203.
11. Sherman FS. Steady viscometric flows. In: Corrigan J, Morriss JM, editors. *Viscous flow*. Singapore: McGraw-Hill; 1990. p 149-150.
12. Nordon RE. Uniform shear elution affinity cell separation [doctoral thesis]. Sydney: Graduate School of Biomedical Engineering, University of New South Wales; 1994.
13. Granger J, Dodds J, Midoux N. Laminar flow in channels with porous walls. *Chem Eng J* 1989;42:193-204.
14. Koeffler HP, Billing R, Lusic AJ, et al. An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). *Blood* 1980;56:265-273.
15. Doheny JG, Jervis EJ, Guarna MM, et al. Cellulose as an inert matrix for presenting cytokines to target cells: production and properties of a stem cell factor-cellulose-binding domain fusion protein. *Biochem J* 1999;339(pt 2):429-434.
16. Nordon RE, Haylock DN, Gaudry L, Schindhelm K. Hollow-fibre affinity cell separation system for CD34+ cell enrichment. *Cytometry* 1996;24:340-347.
17. Nordon RE. Method and apparatus for culturing cells. PCT/AU/01197. Sydney: Unisearch Ltd; 1999.