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Cell Fractionation and Arrangement on Fibers, Beads, and Surfaces

(immunology/cell culture/lectins/concanavalin A/erythrocytes)

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ABSTRACT **A new method, fiber fractionation, has been used to isolate and separate cells. The cells are adsorbed to fibers covalently coupled to molecules such as antigens, antibodies, and lectins which can bind specifically to cell-surface components. The cells are then removed mechanically by plucking the taut fibers. Alternatively, competitive inhibitors of binding may be used to remove the cells at a lesser rate. Successful fractionations have been achieved by varying the degree of derivatization of the fibers by the lectin concanavalin A. Lymphoid cells have been separated by the use of different antigens coupled to the fibers. The method may also be used for specific fixation and manipulation of viable cell populations in culture. In addition to fibers, beads and surfaces have been specifically derivatized and used to achieve different geometrical arrangements of the cells.**

A number of physical methods for the fractionation of eukaryotic tissues and cells are in current use, but there are few methods utilizing the chemical properties and specificities of the cell surface as a means for both cell fractionation and cell manipulation. Specific methods for the isolation of antibody-forming cells have been developed with various degrees of success (1–3). A more generally applicable method would be of obvious value in cell biology, virology, and immunology.

The main requirements of a chemical approach to the manipulation of cell populations are specificity, general applicability, high yield, and maintenance of cell viability. The requirement for specificity suggests the use of a method employing solid supports coupled to proteins capable of binding to cell-surface components. Subsequent specific dissociation of cells from such supports is limited, however, by the fact that the structures of the surface components are usually not known, nor are they generally available in soluble form for use as competitive inhibitors of cell binding.

In an effort to circumvent some of these difficulties, we have devised a method of fractionation based on the interaction of cell surfaces with chemically derivatized fibers. A variety of molecules such as lectins, antibodies, or anitgens are covalently coupled to the fibers to provide the requisite binding specificity for the cells. Adsorbed cells are removed by plucking the taut fiber, thereby completing the process of fractionation. The fixation technique may also be used with flat surfaces and cells may be made sessile in tissue culture in arrangements that are under control of the investigator. In the present report, we describe preliminary experiments suggesting that fiber fractionation and surface fixation are specific and generally applicable methods for the study of cell populations.

Abbreviations: BSA, bovine-serum albumin (crystallized, Armour Pharmaceutical; Kankakee, Ill.); Con A, concanavalin A; PBS, phosphate-buffered saline, pH 7.4 (8.00 g NaCl, 0.20 g KCl, 0.20 g KH_2PO_4, 0.15 g Na_2HPO_4/liter).

MATERIALS AND METHODS

Cell suspensions

Cells were obtained from BALB/c mice (Jackson Labs, Bar Harbor, Me.), NCS mice (Rockefeller University, New York, N.Y.) or New Zealand White rabbits. Erythrocytes were washed twice in phosphate-buffered saline (PBS) before use. Peripheral lymphocytes were isolated by the method of Coulson and Chalmers (4). Thymocytes and spleen cells were prepared by teasing the organs through a wire mesh into PBS or Hank's balanced-salt solution (GIBCO, Grand Island, N.Y.). Aggregates were removed by low-speed centrifugation and the cell suspensions were washed twice in the medium. Viability, determined by the exclusion of vital stain, exceeded 90% for thymocytes and 70% for spleen cells.

Immunization

BALB/c mice were injected intraperitoneally with 100 μg of Dnp_{38}-bovine γG immunoglobulin (Armour, Fraction II), 100 μg of $tosyl_{30}$-bovine-serum albumin (Armour; BSA) or 1 mg of BSA in complete Freund's adjuvant (5). The subscripts indicate the mole ratio of hapten to protein in the antigen conjugates. After 2–4 weeks, secondary injections of 50 μg of antigen in PBS were administered intraperitoneally. Rabbits were immunized intramuscularly with 5 mg of antigen in complete Freund's adjuvant and were given a secondary intravenous injection of 100 μg of antigen in PBS. Spleens were removed 4–6 days after the secondary immunization.

Preparation of derivatized fibers, plates, and beads

Transparent nylon monofilament (size 50 sewing nylon; Dyno Merchandise Corp., Elmhurst, N.Y.), was strung into polyethylene collars (cut from hollow S-6 stoppers, Mallinckrodt, N.Y., N.Y.). These fit snugly into 35 × 10 mm Petri dishes (NUNC, Vanguard International Inc., Red Bank, N.J.) and hold the nylon fibers under tension (Fig. 1). This arrangement greatly facilitates the handling and subsequent use of the fibers. Surface contaminants were removed by 10-min extractions of the strung fibers, first with petroleum ether and then with carbon tetrachloride. In order to increase the reactivity of the nylon fibers, they were partially hydrolyzed with 3 N HCl for 30 min at room temperature (6). After thorough rinsing in H_2O, the fibers were placed in a Petri dish containing 2 ml of a solution of either Concanavalin A (Con A) or various antigens in 0.15 M NaCl (pH 6.0). Protein concentrations ranged from 0.05 mg/ml to 5 mg/ml; specific values are indicated for each experiment. A water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate (Aldrich Chemical, Milwaukee, Wis.), was used to couple protein covalently to the nylon (7). 2 ml of this reagent in 0.15 M NaCl (pH 6.0) at a carbodiimide to protein

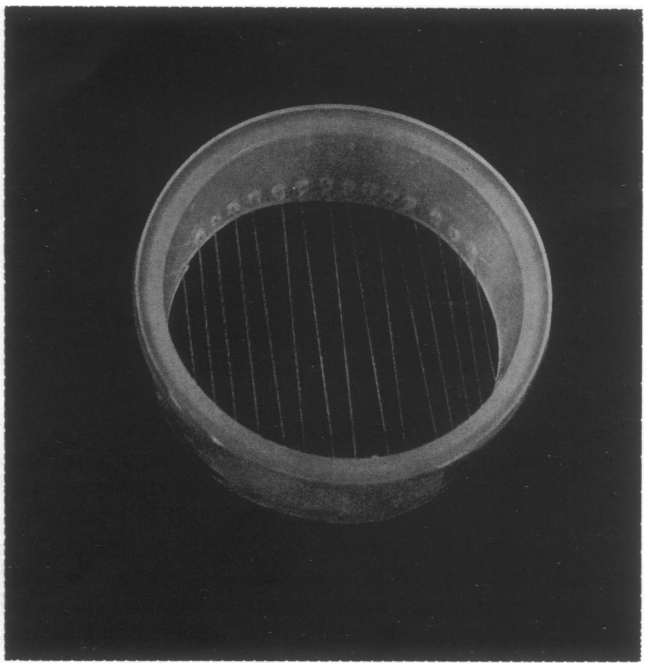

FIG. 1. Petri dish containing polyethylene collar strung with nylon monofilament.

ratio of 5:1 (w/w) was added to the Petri dishes and the reaction mixture was shaken at room temperature for 30 min. The polyethylene collars were washed, transferred to fresh Petri dishes, and stored overnight in PBS before use. In order to determine the extent of coupling at particular Con A concentrations (Fig. 2), Con A prepared by the procedure of Wang *et al.* (8) and labeled with ^{63}Ni by the method of Inbar and Sachs (9) was used for derivatization.

The coupling procedure described above was also used for the direct derivatization of the surface of Petri dishes containing no nylon fibers. The extractions with petroleum ether and carbon tetrachloride, and the partial hydrolysis with HCl were omitted.

Agarose 6B (Pharmacia, Uppsala, Sweden) was covalently coupled to Con A by a modification of the technique of Porath

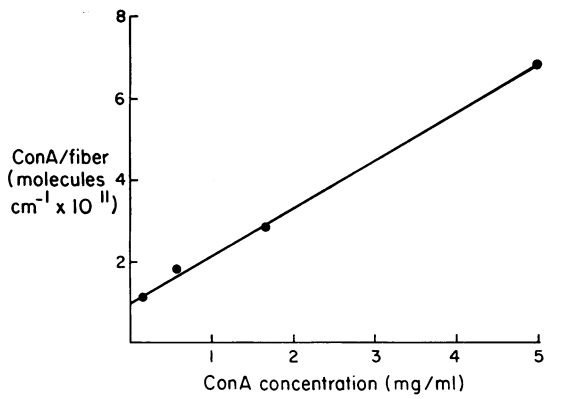

FIG. 2. Derivatization of nylon fibers with [^{63}Ni]Con A. Derivatized fibers were extracted with 1 N acetic acid for 12 hr to solubilize the ^{63}Ni, which was assayed by liquid scintillation spectrometry (9). The extent of Con A coupling was calculated from the length of fiber analyzed and the specific activity of the ^{63}Ni-labeled Con A; the molecular weight of Con A at pH 6.0 was assumed to be 54,000 (8).

(10). The beads were suspended in an equal volume of H_2O and adjusted to pH 10.5 with 0.1 N NaOH. Cyanogen bromide, 200 mg in 160 ml of H_2O, was slowly added at room temperature with stirring to 100 ml of the Agarose suspension. The reaction mixture was maintained at pH 10.0–10.5 for 8–10 min, washed on a Buchner funnel with cold H_2O, transferred to a beaker in a volume of 50 ml, and diluted with an equal volume of 0.2 M potassium phosphate buffer (pH 6.5). Con A, 1.0–1.5 mg/ml in 0.15 M NaCl, was added to give a volume of 150 ml. The reaction mixture was gently stirred overnight at 4°C before the beads were washed in 0.15 M NaCl. Microscopic examination of the derivatized beads showed that they were undamaged.

Cell binding and removal

Cells were bound to derivatized nylon filaments by the addition of 10^7–10^8 cells in 4 ml to the dish with the nylon fibers. The dishes were placed on a platform shaker (80 oscillations/minute) at 21°C for 15–180 min, after which unbound cells were removed by complete immersion of the dish in a series of larger vessels containing the medium. During this and all subsequent procedures, care was taken not to remove the fibers from the liquid because removal resulted in the release and death of the cells. Cells were recovered from fibers either chemically, by incubation in solutions containing a competitive inhibitor, or mechanically, by gently plucking the fibers once at each end.

Cells were bound directly to the surface of derivatized Petri dishes containing no fibers by addition of 4 ml of a cell suspension (10^7–10^8 cells/ml) to the dishes and incubation at room temperature for 10 min. Unbound cells were removed by immersion of the dish in PBS.

For binding of cells to beads, Con A-coupled Agarose, mixed 1:1 with untreated Agarose, was packed into 4.0 × 0.7 cm columns and washed with 0.15 M NaCl. An erythrocyte suspension containing 10^8 cells in 0.15 M NaCl was loaded on the

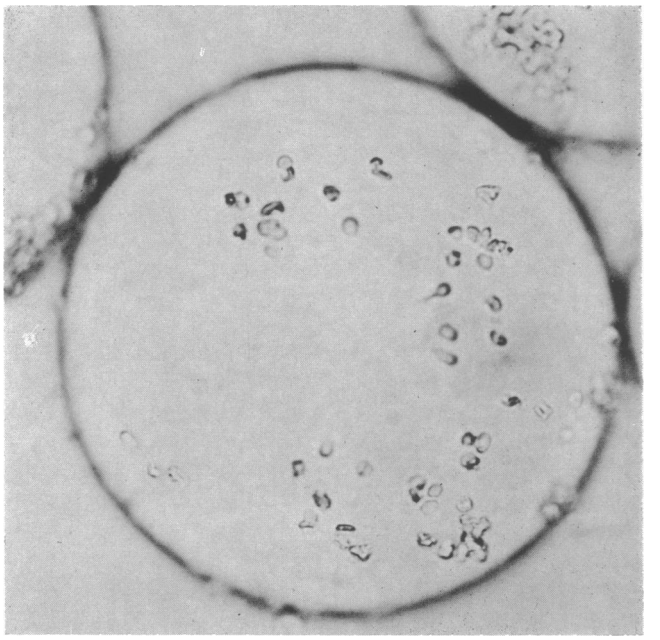

FIG. 3. Mouse erythrocytes bound to a Con A-derivatized Agarose 6B bead. (×200 magnification; bright-field microscopy).

columns. The columns were then washed with 10 ml of 0.15 M NaCl. Adherent cells were eluted with solutions as indicated in *Results*. 1-ml fractions were collected and cell concentrations were determined by counting in a hemocytometer.

RESULTS

A series of experiments was conducted on Con A–Agarose beads to define conditions for cell binding and removal. Beads of derivatized Agarose-bound mouse erythrocytes, thymocytes, and lymphocytes (Fig. 3); untreated Agarose, Agarose activated with CNBr but not coupled to protein, or Agarose coupled to BSA failed to bind any cells. Individual Con A–Agarose beads bound an average of 100 cells, consistent with the finding that a column containing 5×10^5 derivatized beads bound about 5×10^7 erythrocytes. Pretreatment of columns of Con A–Agarose beads with reagents known to inhibit Con A binding, such as α-methyl-D-mannoside, D-glucose (11), or EDTA prevented binding of the cells. The EDTA-treated columns could be reactivated with $NiCl_2$ and $CaCl_2$ (12). These results indicate that the initial adherence of the cells to Con A–Agarose was due to the specific reaction of the lectin with receptor sites on the cell surface.

Once the cells were bound to the columns, however, it was difficult to remove them. Flowing isotonic solutions containing 0.001 M–0.3 M concentrations of the inhibitors α-methylmannoside, D-glucose, or D-melezitose, or 5 mM EDTA, failed to remove thymocytes or erythrocytes. Treatment of columns containing bound cells with dilute solutions of trypsin, chymotrypsin, or sodium metaperiodate also had no effect despite the fact that similar treatment of the columns before cell binding destroyed their capacity to bind cells. If, however, the columns saturated with erythrocytes were incubated without flow in 0.3 M α-methylmannoside at pH 7.4 for 2 hr, 90% of the bound cells were recovered upon restarting the elution.

Immediate removal of adherent erythrocytes was achieved when the total milliosmolarity (mOsM) of inhibitor solutions was less than 310 mOsM. As shown in Table 1, 2 ml of a 150 mOsM solution, consisting of 0.05 M NaCl and 0.05 M α-methylmannoside (pH 7.4) released 85–90% of the cells with no apparent damage. If the total milliosmolarity was raised above 150 mOsM, fewer cells were removed. It was found that at least 50 mM NaCl and 5 mM α-methylmannoside were needed to achieve satisfactory elution (Table 1). As discussed below, these conditions were found to be useful in removing erythrocytes from fibers.

TABLE 1. *Elution of erythrocytes from Con A–Agarose columns as a function of milliosmolarity and α-methylmannoside and salt concentration*

Total milliosmolarity*	Millimolar concentration: α-Methyl-mannoside	Millimolar concentration: [NaCl]	% Cell recovery
150	50	50	85–90
150	5	50	85–90
150	1	50	0
150	50	35	30–40
150	50	25	0
200	50	50	20–25
250	50	50	15–20
300	50	50	0

* Where necessary, the total milliosmolarity was adjusted by addition of galactose, which does not bind to Con A (11).

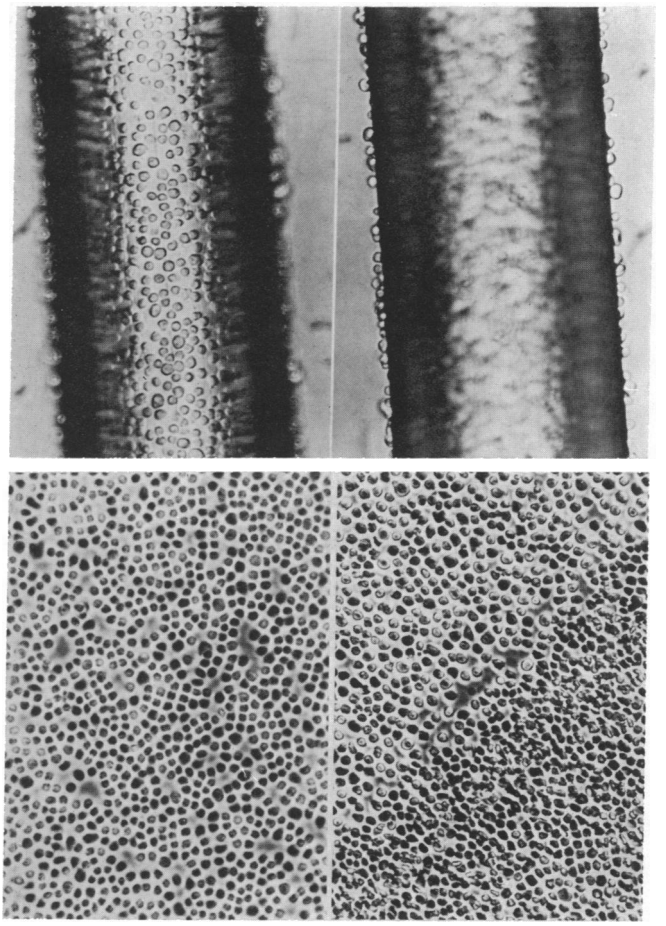

FIG. 4. (*a*) *Left:* Mouse thymocytes bound to a Con A-derivatized nylon fiber. The field was focused on the face of the fiber at ×200 magnification by bright-field microscopy. *Right:* Mouse thymocytes bound to a Con A-derivatized nylon fiber. The field was focused on the edge of the fiber at ×200 magnification by bright-field microscopy. (*b*) *Left:* Mouse thymocytes bound to the surface of a Con A-derivatized Petri dish at ×200 magnification by phase-contrast microscopy. *Right:* Mouse thymocytes and erythrocytes coupled to different portions of a Con A-derivatized Petri dish. Erythrocytes were applied to a section of the surface in saturating numbers, and unbound cells were removed by washing with PBS. The dish was filled with a saturating number of thymocytes and unbound cells were removed. The field shows a region where the two cell types are adjacent to each other (×200 magnification by phase-contrast microscopy).

Specific binding and release of mouse erythrocytes could also be achieved with Sephadex G-100 beads that were noncovalently coupled to Con A. Beads were incubated with 1.0 mg/ml of Con A in PBS (ph 7.4) for 10 min at 21°C, washed in PBS, and incubated with an erythrocyte suspension (10^7 cells/ml). Adherent cells could be quantitatively released from the beads by a 10-min incubation in 0.1 M α-methylmannoside (pH 7.4).

Fiber fractionation and surface fixation of cells

Mouse thymocytes and erythocytes bound to nylon monofilaments and Petri dishes that had been derivatized with Con A are shown in Fig. 4. The cells are firmly attached to the

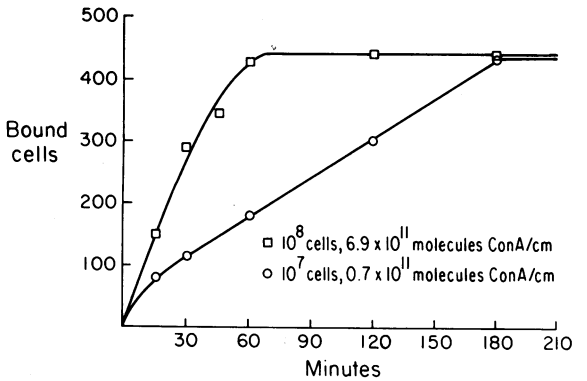

FIG. 5. Extent of thymocyte binding to Con A fibers using standard conditions of mixing and shaking (see *Methods*). The number of bound cells was determined by microscopic examination of a field 1 mm in diameter. Values represent the average of 5 determinations within each dish.

supports and lie in an evenly spaced monolayer. The binding did not reduce cell viability, nor did it distort the shape of the cells. Thymocytes bound to derivatized dishes survived for 72 hr in tissue culture. It was possible to fix two-cell populations so that they were adjacent to each other on the surface of the same Con A-derivatized Petri dish (Fig. 4*b*).

In contrast to beads in columns, the process of attachment and removal of cells to fibers could be observed directly. The fibers were saturated with adherent cells in 60–180 min depending upon the degree of Con A derivatization and the cell concentration used for binding (Fig. 5). Addition of 0.01 M α-methylmannoside before exposure to the cell suspension inhibited the binding of the cells to fibers and dishes. No binding of thymocytes was observed when underivatized fibers or fibers coupled to BSA, γG immunoglobulin, or ovalbumin were used.

The effect of the extent of Con A substitution on the number of cells bound is shown in Fig. 6. Under saturating conditions, thymocytes and erythocytes exhibited markedly different binding thresholds. For example, a fiber with 7×10^{11} Con A molecules/cm bound both erythrocytes and thymocytes, while a fiber having 1×10^{11} molecules/cm bound only

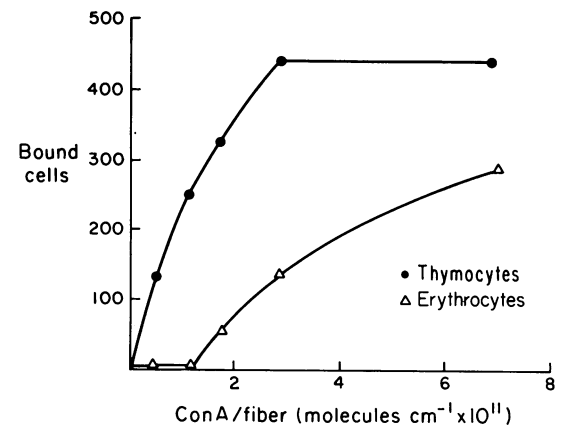

FIG. 6. Binding of erythrocytes and thymocytes to different fibers as a function of the number of Con A molecules/cm of nylon fiber. 2×10^8 cells in 4 ml of PBS were incubated with different fibers for 120 min with standard mixing conditions. Cells were counted as described in the legend of Fig. 5.

thymocytes. A 2-hr incubation period with 0.01–0.3 M α-methylmannoside, was required for 90% recovery of erythrocytes from fibers with 7×10^{11} Con A molecules/cm. Erythrocytes could be instantaneously released, however, with a hypotonic solution (150 mOsM) containing 0.05 M α-methylmannoside and 0.05 M NaCl.

In order to obtain a high yield of viable thymocytes, the fibers with 1×10^{11} Con A molecules/cm were used. These fibers were incubated for 60 min with a cell suspension containing 5×10^7 erythrocytes and 5×10^7 thymocytes/ml of PBS. After the fibers were washed, the cells on the fibers were exclusively thymocytes. Each dish contained 10^6 cells, which could be recovered quantitatively by plucking the fibers in heat-inactivated fetal-calf serum diluted 1:10 with PBS. Direct visual inspection of the process of cellular removal showed that the transverse components of the mechanical vibration induced by plucking projected the bound cells into the medium. After incubation in the medium containing fetal-calf serum for 1 hr at 37°C, the cells that had been removed

TABLE 2. *Isolation of spleen cell populations by fiber fractionation*

Antigen coupled to fiber*	Immunogen†	Inhibitor‡	Number of bound cells¶ Expt. 1	Expt. 2
Dnp-BSA	Dnp-γG immunoglobulin	0	478	284
Dnp-BSA	Dnp-γG immunoglobulin	Dnp	81	67
Dnp-BSA	Dnp-γG immunoglobulin	Tosyl	491	253
Dnp-BSA	Unimmunized	0	156	132
Dnp-BSA	Unimmunized	Dnp	63	54
Dnp-BSA	Unimmunized	Tosyl	179	115
Tosyl-BSA	Tosyl-BSA	0	210	297
Tosyl-BSA	Tosyl-BSA	Tosyl	78	36
Tosyl-BSA	Tosyl-BSA	Dnp	209	261
Tosyl-BSA	Unimmunized	0	127	130
Tosyl-BSA	Unimmunized	Tosyl	67	63
Tosyl-BSA	Unimmunized	Dnp	115	105
BSA	BSA	0	112	85
BSA	BSA	BSA	37	18
BSA	BSA	Dnp	116	—
BSA	Unimmunized	0	58	—
BSA	Unimmunized	BSA	38	—
BSA	Unimmunized	Dnp	58	—

* Dnp: Dnp_8-BSA derivatized nylon; tosyl: $tosyl_{20}$-BSA derivatized nylon; BSA: BSA-derivatized nylon. The coupling reaction was conducted at 0.5 mg/ml of each antigen.

† Dnp: immunization with Dnp_{38}-G immunoglobulin; tosyl: immunization with $tosyl_{30}$-BSA; BSA: immunization with BSA.

‡ 100 μg/ml of Dnp_8-BSA and $tosyl_{20}$-BSA; 50 μg/ml of *N*-Dnp-ϵ-Lysine (Sigma Chemical, St. Louis, Mo.) was used in some experiments with similar results; 500 μg/ml of BSA.

¶ Numbers represent total cells bound to the edge (Fig. 4*a*) of a 2.5-cm fiber segment. 1×10^8 cells in 4 ml of Hank's solution were added to the dishes; other conditions were the same as described for Con A fibers. In all experiments, the background in the presence of the inhibitory antigen was about 50 cells/2.5-cm fiber segment. Values represent the average of 5 determinations within each dish. All experiments were done with mice, except for tosyl Expt. 2, which was done with rabbits.

mechanically from the fibers were 80–90% viable. The number of cells excluding dye increased from 30 to 90% during the course of the incubation; incubation in PBS alone or substitution of 8% BSA for the fetal-calf serum led to reduced cell viability.

Although all cells could be removed from any Con A fiber by plucking, few viable thymocytes could be obtained by this method when derivatized fibers having more than 2×10^{11} Con A molecules/cm were used. Cells removed from these heavily derivatized fibers had visible breaks in their membranes that resulted in the loss of cytoplasm. The fibers had a diminished capacity for thymocyte binding after plucking, which suggests that pieces of cell membrane may have remained on the Con A–nylon.

Fiber fractionation was applied to spleen cells from specifically immunized mice and rabbits, by the use of nylon fibers derivatized with different antigens. Antigen-coupled fibers always bound more spleen cells from specifically immunized animals than from unimmunized animals (Table 2). Addition of the soluble antigen before addition of the cells eliminated this difference by reduction of the binding of both cell populations to background levels. The antigen-specific binding as determined by the fiber-dish assay was also reduced by prior absorption with increasing amounts of 1-cm pieces of fiber derivatized with the same antigen. The number of cells bound to a strung fiber after absorption decreased in proportion to the number of loose fibers added until background levels were reached. Less than 1% of the cells were removed by this procedure. The specificity of binding was also demonstrated by cross-inhibition studies: tosyl derivatives did not inhibit Dnp-specific binding appreciably, nor did Dnp derivatives inhibit tosyl-specific binding. In contrast to spleen cells, thymocytes from immunized and unimmunized mice did not bind to antigen-derivatized fibers.

DISCUSSION

The present experiments suggest several novel and versatile approaches to the specific fractionation and manipulation of cell populations. The actual process of cellular adhesion may be observed under the microscope, various cells may be specifically bound, and they may be removed from the fibers quantitatively by chemical or mechanical means. Removal of bound cells from the fibers by the addition of molecules that compete for the binding sites is obviously limited, however, to those cases in which the inhibitor is known. This limitation of affinity chromatography (13) may be circumvented by carefully controlled mechanical removal of cells from derivatized fibers.

The fiber fractionation technique is applicable to a variety of cells. Using the lectin Con A as the binding agent, we have been able to fractionate a mixture of thymocytes and erythrocytes, and with antigens as the binding agents, a specific isolation of immune cells was achieved. In the latter case, cell binding could be inhibited by the presence of soluble antigens. Relatively large numbers of cells from unimmunized animals were bound to both Dnp- and tosyl-derivatized fibers and this binding was also shown to be specifically prevented by the presence of free antigens. Analysis of rosette-formation by cells from the spleens of unimmunized animals has also shown relatively high numbers of reactive cells (14, 15). The use of antigen-derivatized fibers provides a possible approach for the quantitative study of clones of committed cells in immunized and unimmunized animals; studies with other antigens are currently under way.

Although the initial fixation of cells to Con A–Agarose beads was specific, quantitative elution of bound cells by a competitive inhibitor was difficult to achieve. Similar observations have been made by Wigzell *et al.* (1) using antigen-coupled acrylic beads. The cause of this phenomenon is not known, but the present experiments rule out mechanical trapping of cells by the Agarose columns or beads themselves. Cells were released from Con A–beads and fibers under hypotonic conditions, where the cell membranes are likely to be stretched. This suggests that, after being specifically bound, the cell membrane may interact with the surface of the bead or fiber to form secondary adhesions that are broken only when the cell is distorted.

The geometry and manipulability of derivatized fibers, fiber meshes, and surfaces allows various schemes to be used in cell-fractionation experiments. Successive portions of a fiber can be substituted with different binding molecules and various arrangements of different cell types in tissue culture may be obtained. This is also true for procedures using large surface areas of culture dishes that have been derivatized with lectins, antigens, or antibodies (Fig. 4*b*). Portions of cell membranes that remain behind after mechanical removal of the cells from the derivatized surface may be recovered and analyzed. Investigations of cell mobility, membrane interactions, pseudotissue formation, and cell cooperation are possible. Cells may be geometrically arranged in predetermined patterns, mechanically transported as groups, juxtaposed, and then physically removed for later analysis. Attachment of cells by lectins or antibodies to fibers that already possess covalently attached enzymes or hormones provides an additional means for the study of cell-surface biochemistry both *in vitro* and *in vivo*. We are currently studying the possibility of building a machine for the automatic fiber fractionation of large numbers of cells.

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