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Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter

(fluorescent microspheres/monoclonal antibodies/immunoglobulin allotypes/autofluorescence)

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ABSTRACT **Myeloma–spleen cell hybrids (hybridomas) producing antibody to mouse immunoglobulin allotypes have been labeled with fluorescent microspheres coupled with myeloma protein antigens. The ratio of specific to nonspecific microsphere binding by viable hybridoma cells was about 100:1. By using a modified fluorescence-activated cell sorter (FACS), selected hybridoma cells in a mixture have been sorted individually into media in microculture wells, where, with thymocyte feeder cells, they developed into clones producing a desired monoclonal antibody. Viable cells were selected by measurement of their light scattering and autofluorescence properties. Rare antibody-producing clones were obtained without laborious screening and repeated subculturing. This technique should expand the range of monoclonal antibodies readily obtained from hybridomas and greatly facilitate the process of obtaining desired hybridomas.**

The recent development of myeloma hybrid cell lines (hybridomas) producing antibodies with desired reactivities is revolutionizing the production of immunospecific reagents (1). Such lines allow indefinite production of large quantities of homogeneous (monoclonal) antibody specific for any single antigenic determinant of an immunizing antigen despite the overall heterogeneity of the antibody response to that antigen. Cloning and selecting lines with desired reactivities, however, is currently often a laborious procedure that constitutes the major limitation to rapid establishment of a wide range of lines producing antibodies useful for research and medicine.

The current selection procedures rely on dispersion of cells in soft agar or on limiting dilution microculture for physical isolation of the hybrid clones after hybridization. Desired hybrids are identified by screening for production of antibody after clones grow (1–3). For antigens that lend themselves to a simple hemolytic plaque assay, a high selection efficiency is possible. But for many antigens—e.g., most protein antigens, allotypes, impure mixtures of naturally occurring antigens—not readily adapted to a plaque assay, culture supernates are assayed for antibody. Clearly, screening more than a few hundred individual culture supernates is not practical. Aside from the difficulties in screening large numbers of isolates to find the few containing desired clones, these methods are often unsatisfactory because desired clones may be lost due to overgrowth of the culture by unwanted clones or nonproducing variants of the desired clone. Therefore we have developed means to label antibody-producing hybrid cells selectively with fluorescent tagged antigen. By using a fluorescence-activated cell sorter (FACS) and this labeling, 10^3 cells can be screened per second and desired ones can be isolated and cloned on the basis of their cell surface-associated antibody. Recently, other workers have reported using a FACS and fluorescein-tagged antibodies to immunoglobulin (Ig) classes to select for myeloma variants secreting immunoglobulins of different classes (4).

Our initial attempts to label cells with directly fluoresceinated antigen were disappointing due to the small amount of antigen bound, but the use of fluorescent microspheres (5–7) to which we conjugated our antigens overcame this sensitivity problem and allowed specific staining of hybridoma lines.

With mouse myeloma protein-coupled microspheres we could easily detect and isolate hybridomas producing monoclonal antibodies reactive with allotypic or other determinants on the myeloma proteins. To directly clone selected cells, we modified the FACS sorting system to enable deposition of single cells under sterile conditions into microculture wells. We have been able to isolate specific antibody-producing hybrids quite efficiently even when they constitute a very small fraction of the cell mixture.

MATERIALS AND METHODS

Hybrid Cells. The hybrid cells used in this work were produced by fusion of the NS-1 variant of the mouse P3 (MOPC-21) myeloma line (8) with spleen cells from mice immunized with the antigen of interest. Details of hybridization procedures are described elsewhere (3, 9, 10). The production and characterization of hybrids producing antibody to mouse immunoglobulin allotypes and to molecules in the major histocompatibility complex, including clones Ig(1b)2.4 and 11-5.2 used in this work, have been described (10). The cells were cultured in RPMI-1640 medium (GIBCO) containing 15% fetal calf serum. Incubation was at 37°C in an atmosphere containing 7% CO_2.

Four distinct cell populations were used in this work.

(*i*) Clone Ig(1b)2.4 secretes IgG_3 antibody with Ig-1b (IgG_{2a} of the *b* allotype) reactivity. It was produced through immunization of BALB/c mice with C57BL/10 (Ig^b) *Bordetella pertussis*–anti-*B. pertussis* complexes. A subclone of this, Ig(1b)2.4.12, was also used.

(*ii*) Clone 11-5.2 produces antibody to I-A^k, the product of a locus of the *I*-A^k halotype of the murine major histocompatibility complex. The spleen cell donor was a BALB/c (H-2^d, Ig^a) immunized with CKB (H-2^k, Ig^b) spleen cells.

(*iii*) Uncloned population 20-6 was derived from an immunization of SJL mice with BALB/c Ig (*a* allotype) in the form of *B. pertussis*–anti-*B. pertussis* complexes. It secretes Ig-1b (IgG_{2a}) antibody with reactivity to Ig-1a. Clone 20-6.B6 was also used in this work.

(*iv*) Uncloned population 20-9 was derived from the same hybridization as 20-6. It produces antibody reacting with BALB/c Ig and with MOPC-21 (IgG_1, *a* allotype) myeloma protein.

Antigen-Coupled Microspheres. Polymeric microspheres

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Abbreviation: FACS, fluorescence-activated cell sorter.

of 0.783-μm diameter were obtained from Polysciences, Inc. (no. 7766, Warrington, PA). They are blue-green fluorescent (excitation peak at 465 nm and emission peak at 485 nm) and have carboxyl groups on their surface.

Myeloma proteins for use as Ig allotype antigens were obtained by affinity chromatography on Sepharose-*Staphylococcus aureus* protein A (Pharmacia) (11).

Myeloma proteins GPC-8 (Ig-1a) and C.BPC-101 (Ig-1b) were coupled to the fluorescent microspheres by a carbodiimide reaction (12). Roughly 40% of added ^{125}I-labeled myeloma protein (originally 5 mg per 100 mg of microspheres) was coupled to the microspheres, indicating about 25,000 IgG molecules per microsphere. The microspheres were dispersed by sonication before use.

Cell Labeling with Antigen-Coupled Microspheres. Hybridoma cells were washed and mixed with microspheres in 24-well culture trays (Costar no. 3524, flat-bottomed wells, 16 mm in diameter, Cambridge, MA). Each well normally contained 5×10^5 cells and 100 μg of microspheres in 1 ml of RPMI-1640 with 15% non-heat-inactivated fetal calf serum. The trays were centrifuged at $500 \times g$ for 15 min, sedimenting the cells and microspheres together on the tray bottom in approximately a monolayer. After 15–30 min at room temperature the cells were washed to remove unbound microspheres by centrifugation at $60 \times g$ through a layer of fetal calf serum. Mixing the cells and antigen-coupled microspheres in suspension (i.e., without centrifugation) even at much higher concentrations than specified above results in almost no specific binding of the microspheres.

Assay for Secreted Antibody. Antibody binding to myeloma protein C.BPC-101 or GPC-8 was measured in a solid-phase radioimmunoassay (10,13).

Viable Cell Counting. Routine total and viable cell counting was done after staining with a mixture of the nucleic acid-specific dyes acridine orange and ethidium bromide. A stock solution containing each dye at 1 μg/ml in saline was mixed 1:1 with the cell suspension. Acridine orange enters living cells, giving the nuclei a green fluorescence. Ethidium bromide is excluded by intact hybridoma cell membranes but stains the nuclei of dead cells orange fluorescent. These dyes can be used in conjunction with fluorescent microsphere labeling. Both dyes are highly mutagenic (14) and should be handled with care.

Cell Sample Analysis and Sorting. Quantitative fluorescence analysis and cell sorting (15) were performed using a FACS-II cell sorter (Becton Dickinson FACS Division, Mountain View, CA). It measures forward-angle light scatter and two channels of fluorescence on a cell-by-cell basis. It was also employed to sort one viable cell per well in 96-well plates (cloning) on the basis of light scattering and fluorescence properties of the cell. Use of the FACS-II for cloning involved modifications that are described below.

FACS measurements were made using the 488-nm argon ion laser line at a power of 0.40 W. Long-pass filters were used to block scattered laser light while passing fluorescent light. The laser line and filter combination used are far from the optimum for exciting and detecting the microsphere fluorescence, but, because the fluorescence of single microspheres was easily measured, there was no need for optimization. The fluorescent light was divided between two photomultiplier tube detectors by a chromatic reflector (LP580, no. 466305, Zeiss), which reflects light with wavelengths shorter than 580 nm and passes longer-wavelength light. The two fluorescence signals are combined linearly to yield independent quantitative measurement of two fluorescent signals from each cell (16).

RESULTS

Specificity of Antigen-Coupled Microsphere Binding. Fig. 1 shows the specificity of binding of antigen-coupled microspheres to viable cells of two clonally derived hybrid cell lines. One, 20-6.B6, produces antibody reactive with determinants on the BALB/c IgG_{2a} myeloma protein GPC-8 (which carries Ig-1a allotype determinants). The other line, Ig(1b)2.4.12, produces antibody reactive with Ig-1b allotypic determinants on the CB.20 IgG_{2a} myeloma protein C.BPC-101. As the figure shows, each cell line specifically binds microspheres coupled to its reactive antigen; i.e., the anti-Ig-1a line binds an average of 44 GPC-8-coupled microspheres per cell but only 0.4 C.BPC-101 microspheres, and the anti-Ig-1b line binds 21 C.BPC-101 microspheres per cell but only 0.2 GPC-8 microspheres. Only 4.7% of the Ig(1b)2.4.12 cells carried even one GPC-8 microsphere. An expansion of the abscissa scale of the binding profile of clone 20-6.B6 with C.BPC-101 microspheres (Fig. 1 *right*) shows the clear distinction between totally unlabeled cells and cells bearing one or two microspheres.

Selection of Viable Hybrid Cells. Dead cells and nonnucleated cellular debris can bind microspheres nonspecifically. To identify viable and specific antigen-binding cells with the FACS, it is therefore important to use both viability and antigen-coupled microsphere binding as criteria for selection.

Forward-angle light scattering measurements alone can distinguish live from dead cells in certain types of cell suspensions—e.g., splenic lymphocytes (17)—but with hybridoma

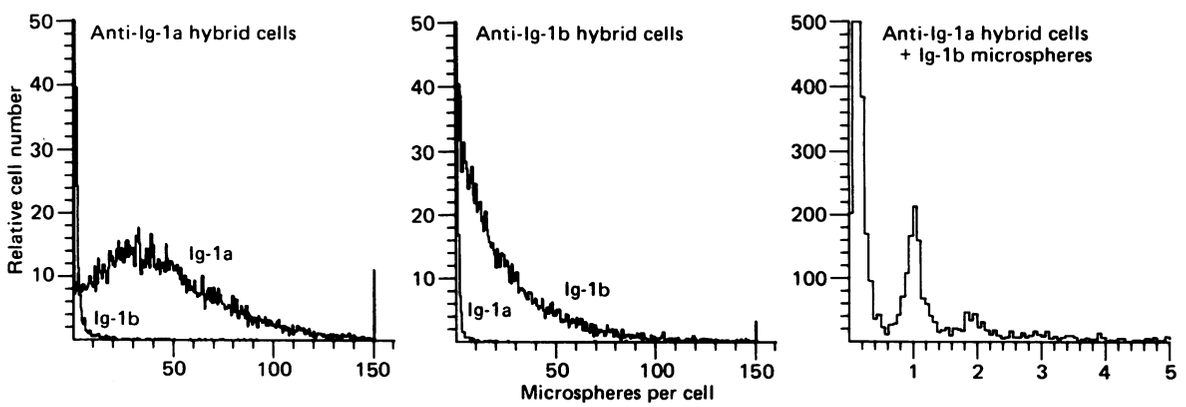

FIG. 1. Specific binding of IgG antigen-coupled microspheres by two hybrid clones. (*Left*) Binding profiles of clone 20-6.B6 (anti-Ig-1a producer) with GPC-8 (Ig-1a) microspheres and C.BPC-101 (Ig-1b) microspheres. (*Center*) Binding profiles of clone Ig-(1b)2.4.12 (anti-Ig-1b producer) with the same microspheres. (*Right*) Details of a binding profile at higher amplification for 20-6.B6 cells treated with C.BPC-101 microspheres.

lines such light scattering measurements are not sufficient to clearly distinguish live from dead cells. However, the dead cells exhibit more red autofluorescence than do live hybridoma cells. Light scattering and red autofluorescence criteria, taken together, define viable hybridoma cells very well. Signals from the two fluorescence detectors of the FACS-II are transformed by a two-color compensation network (16) to obtain independent signals for cell (red) autofluorescence and microsphere (green) fluorescence. Fig. 2 shows a dot-plot of forward-angle light scattering vs. red autofluorescence for a hybridoma cell sample. The viable cell population is clearly discriminated from dead cells and debris. Viable cells defined by the scatter and autofluorescence (red) signals can then be sorted according to the number of microspheres bound using the other (green) fluorescence channel of the FACS.

Direct Cloning Using the FACS. Because the FACS operates on a cell-by-cell basis, an efficient way to obtain desired hybridoma clones would be to deflect one viable cell binding a selected number of microspheres per (micro) culture. We have (reversibly) modified the FACS to accomplish this. The modifying electronics constitute a refinement of that used previously for sorting single cells onto slides (18). The mechanics are diagrammed in Fig. 3. Undeflected drops from the liquid jet are intercepted and aspirated away. After a signal from the FACS operator one, and only one, cell meeting preset light scatter, autofluorescence, and microsphere fluorescence criteria is deflected into a well of a 96-well microculture tray. The wells of the tray are prefilled with 0.1 ml of culture medium containing 10^6 thymocytes as feeder cells (19). Between deflections, the culture tray is moved manually to target the next well. Asepsis is readily maintained.

Recovery of Rare Antibody-Producing Cells. We performed several experiments as practical demonstrations of the specificity of hybrid cell labeling with antigen-coupled microspheres and of the ability of the FACS to select and directly clone rare cells. In each case, cells producing antibody with reactivity for a particular myeloma protein were mixed, at a frequency of 1 in 500, with cells producing some other antibody. The rare cells were then recovered by labeling with appropriate antigen-coupled microspheres and sorting the brightly labeled cells with the FACS.

In one such experiment, Ig(1b)2.4 cells producing antibody to Ig-1b were mixed with 11-5.2 cells producing antibody to the I-A^k cell surface antigen. The mixture was labeled with C.BPC-101 (Ig-1b)-coupled microspheres, and the brightest 0.27% of viable cells (those binding more than eight microspheres) were "sorter cloned." Eleven out of 18 clones tested were found to secrete antibody to Ig-1b. This is an enrichment of over 250-fold from the original 1:500 mixture.

To control for possible differences in cloning efficiency between the different cells in the mixture, a similar experiment was performed by using microscopic analysis immediately after sorting rather than culturing to measure the frequency of desired cells in the sorted population. To do this, the rare cells in the mixture were prelabeled with the DNA-specific fluorescent dye Hoechst 33342 (20). The excitation peak of Hoechst 33342 is at 355 nm, and its presence does not affect the measurements of autofluorescence and microsphere labeling that use a 488-nm wavelength laser beam for excitation. Ater sorting, Hoechst 33342-labeled cells were identified and counted, using fluorescence microscopy with near-UV excitation.

Two mixtures were sorted and analyzed this way. In one case the Ig(1b)2.4 cells were prelabeled and mixed with anti-I-A^k-producing cells (clone 11-5.2), and in the other anti-Ig-1a-producing cells (20-6) were prelabeled and mixed with cells (20-9) producing antibody to MOPC-21, an IgG_1 myeloma. The first mixture was then labeled with Ig-1b (C.BPC-101) microspheres, and the second was labeled with Ig-1a (GPC-8) microspheres. In each case the brightest 0.2% of cells were sorted into small wells on a microscope slide. The first mixture yielded 77 Hoechst 33342-labeled cells out of 124, or 62%, and the second gave 29 prelabeled cells out of 85, or 46%. Thus these tests also show an enrichment factor of about 250. Antigen-coupled microsphere labeling and FACS selection should make it possible to obtain desired clones even when appropriate progenitor cells occur at frequencies far below one in 500. The FACS cloning system has been used to select microsphere binding cells constituting 1–5% of the total sample two weeks after hybridization. Numerous specific antibody-producing clones were obtained. Smaller fractions have not yet been encountered.

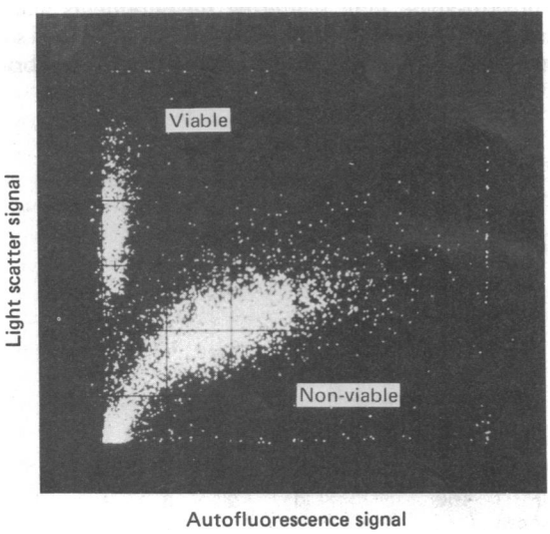

FIG. 2. Dot-plot of forward-angle light scattering vs. autofluorescence for a hybridoma cell sample. In this diagram each dot represents the light scatter and autofluorescence brightness coordinates of a single cell.

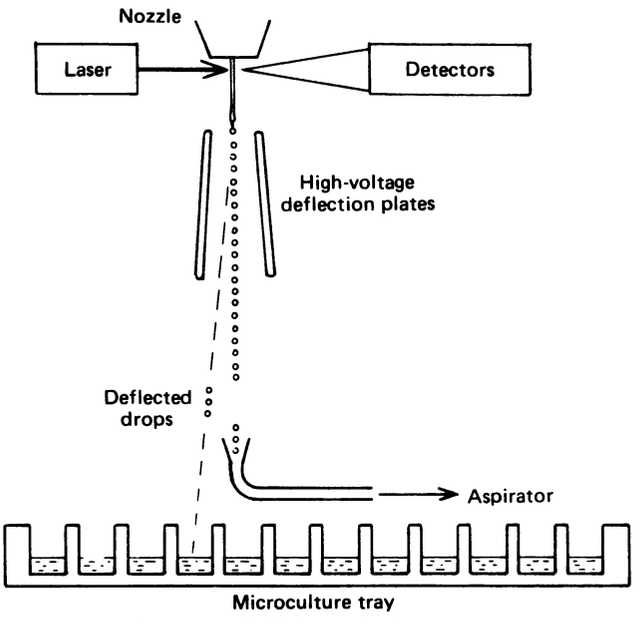

FIG. 3. FACS direct cloning system.

Relation between Antigen-Binding and Antibody Production. All antibody-producing hybrid cell lines we have tested have contained both antigen-binding and non-antigen-binding cells. Sorted antigen-binding cells all give rise to antibody-producing clones (see Table 1, columns 1 and 2) while the non-antigen-binding cells fall into two subgroups, one giving rise to antibody-producing progeny and the other to nonproducing progeny (see Table 1, column 3). These generalizations are illustrated by the data in Table 1 for clone 20-6.B6. The cells were labeled with GPC-8 microspheres, sorted and cloned in three categories: bright (52–70 microspheres bound), dull (3–5 microspheres), and negative (no microspheres). The resulting clones were tested for antibody production and GPC-8 microsphere binding. All of the clones derived from bright or dull progenitor cells secreted antibody to Ig-1a and, where tested, also specifically bound Ig-1a microspheres. Among the antibody-producing clones there were no clear quantitative correlations among (*i*) the number of microspheres bound by the progenitor cell, (*ii*) the rate of antibody secretion by the resulting clone, and (*iii*) the average number of microspheres bound by cells of the clone (unpublished data). Thus only antibody-secreting clones bind antigen-coupled microspheres, but the number bound is not obviously correlated with secretion rate.

Characteristics of Antigen-Coupled Microsphere Binding by Hybridoma Cells. At present we have a very incomplete understanding of the mechanism and characteristics of microsphere binding to cells, but several points have been investigated. First, the binding has been found to be relatively stable: Most bound microspheres are retained by hybrid cells for several hours at room temperature or at 37°C.

Second, microspheres are not internalized by the hybrid cells: Cells from clone 20-6.B6 were labeled with GPC-8 microspheres and then incubated with Pronase (Calbiochem) at 2.5 mg/ml for 30 min at 37°C in serum-free RPMI medium (21). The average number of bound microspheres decreased from 53 to 0.9 per cell. About 80% of the original cells were recovered in viable condition after this treatment. The ability of Pronase to remove microspheres without killing the cells demonstrates that the microspheres were cell-surface bound.

Third, use of antigen-coupled fluorescent microspheres greatly increases cell labeling intensity in comparison to use of soluble fluoresceinated antigen: The average labeling of Ig(1b)2.4.12 cells with optimally fluoresceinated soluble C.BPC-101 was 7% as bright as 1 microsphere while an average of 21 C.BPC-101 microspheres were bound to these cells. The brightness enhancement with microsphere labeling was thus a factor of 300.

Table 1. Secretion and microsphere-binding properties of FACS-sorted clones derived from an anti-Ig-1a-producing clone

Number of microspheres bound by sorted progenitor cell*			Properties of clones derived from sorted progenitors	
52–70 (bright)	3–5 (dull)	0 (negative)	Secretion of anti-Ig-1a	Binding of Ig-1a microspheres
10	10	11	+	+
0	0	12	−	−
0	0	1	±	−
14	14	0	+	Not done

* The cells were from anti-Ig-1a producing clone 20-6.B6; the microspheres were coupled with the Ig-1a myeloma protein GPC-8. Values in the table are number of clones.

DISCUSSION

The strategy we have chosen for obtaining antibody-producing hybrids is to select for antigen-binding cells after fusion. Selection before fusion would not necessarily yield cells in the appropriate developmental stage to give antibody-producing hybrids and would not eliminate the need for selection and cloning after hybridization. Because interesting hybrid cells may be overgrown by others (including their own nonproducing variants), we want to select antigen-binding cells soon after the initial hybridization and clone them directly, recloning as necessary to obtain stable, antibody-producing lines.

We have shown that rare antigen-binding cells can be selected and cloned by using the FACS, provided that a fluorescent label is available that marks the antigen-binding cells with a degree of specificity commensurate with their rarity. Even if the sample contains very few hybrids of the desired type, they can be isolated efficiently by using the techniques reported here. Our experience with Ig antigens coupled to fluorescent microspheres has been encouraging. The sensitivity and specificity of labeling have been excellent and the microspheres remain bound over a period of several hours.

Microsphere labeling profiles in conjunction with measurements of antibody production are yielding information about the cell biology of myeloma hybrids. The subcloning of 20-6.B6, for example, showed that even fresh clones may contain a significant fraction (in this case about 2.6%) of nonlabeling cells that do not secrete active antibody. On the other hand, some nonlabeling cells gave rise to antibody-producing subclones that reproduced the whole range of antigen-coupled microsphere binding observed in the parent clone. If this is not a technical artifact, it implies that there are large differences in antigen-binding capability among the antibody-producing cells on the clone.

The cell sorter cloning technique is quite useful for routine cloning even when there is no fluorescent label to mark cells of particular interest. Viable cells are readily identified by using light scattering and autofluorescence criteria and can be cloned with high efficiency from cultures containing few viable cells. In one case a clone was rescued by sorting out 1 viable cell per 3200 dead cells. This culture could not be saved by other strenuous "farming" efforts. This selective efficiency is also particularly helpful within the first few weeks after cell fusion, when most cells in the cultures are nonviable.

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