Production of Monoclonal Antibodies Using a Secretion Capture Report Web

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We describe a novel method for the production of monoclonal antibodies using a secretion capture report web (SCRW). Following HAT selection in bulk culture, individual hybridomas are encapsulated in biotinylated agarose drops. Antibody secreted by the hybridoma is captured within the agarose drop using an avidin bridge and biotinylated anti-mouse immunoglobulin. The secreted antibody is detected by a fluorescent reporter which can be either a second anti-mouse antibody or an antigen. The binding of the reporter can be quantitated and the desired hybridoma directly cloned by flow cytometry. Multiparameter (i.e., two-color) reporter analysis can also be used to selectively enrich and clone rare hybridomas secreting antibodies directed to unique epitopes. The method allows the characterization of thousands of clones per second and the isolation of hundreds of clones per day.

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It has been nearly 20 years since Kohler and Milstein described a technique for immortalizing antibody producing cells, identifying their products and cloning the cells to obtain monoclonal antibodies (MAbs)[1]. Although this achievement has been repeated countless times by others, the development of a desired MAb has not always been straightforward or easy. Often the inability to obtain a MAb can be traced to a failure to identify the desired antibody-producing hybridomas or to isolate clones of the hybridomas. These two steps in MAb development may require many months of intensive labor and failures at these steps can make the process of isolating a MAb both tedious and time consuming.

The inability to identify and clone the desired hybridomas may result from limitations which are inherent in the technique. Poor outcome has been attributed to the loss of chromosomes by hybridomas especially during HAT selection', contamination of one hybridoma by another in the culture[3–5] and the inability to detect a rare hybridoma within the total population of hybridomas[6]. Traditional analysis of the antibody in accumulated spent medium of hybridoma cultures may result in false identification of a desired hybridoma due to antibody produced by unfused B cells, unstable hybridomas or mixtures of hybridomas. To accurately characterize the antibody from a single hybridoma (i.e., its monoclonal antibody), the hybridoma culture must first be expanded and cryopreserved to guard against loss, cloned by soft agar or limit-dilution techniques, and the supernatant from the clones re-analyzed. Usually for any given fusion this expansion, subcloning, and screening cycle must be repeated for some of the hybridoma cultures.

To overcome the limitations inherent in conventional methodology of MAb development, the ideal method would allow the measurement of antibody secreted by a single hybridoma during a short interval after HAT selection. The method would also allow the identification of potentially rare hybridomas and the ability to clone with high fidelity the desired hybridoma. Weaver et al. described a general method whereby cells could be encapsulated in agarose drops and their secreted product measured using flow cytometry[6]. We have recently improved this technique, referred to here as the Secretion Capture Report Web (SCRW), and demonstrated that it can be used be used to characterize and clone single viable hybridomas'. We demonstrate in this report that the SCRW is an efficient tool for the *de novo* production of MAbs, particularly those of low frequency within a hybridoma population.

Results

In these studies the target antigen was a recombinant fusion protein consisting of the extracellular portion qf the human leukocyte adhesion molecule B7-1 fused with the heavy chain of human IgG[8] (B7Ig). The B7 portion of this fusion protein binds the extracellular portion of its co-receptor, CTLA4 which was also engineered as a human IgG fusion protein* (CTLA4Ig). The fusion proteins were used to examine hybridomas raised to B7Ig in the SCRW and ELISAs. MAbs to B7 were developed as described in Figure 1. After fusion the mixture of mouse splenocytes, myeloma cells and hybridomas was HAT selected in tissue culture flasks. By day 7, myeloma cell viability was $< 1\%$ and the viable hybridomas were isolated by density gradient centrifugation. Viable cells were re-cultured in HAT media and a portion of the culture was cryopreserved. The antibody secreted by single hybridomas was examined using the SCRW, sort-selected as single cells by flow cytometry and culture supernatants of the resulting clones were examined by ELISA.

Analysis of anti-B7Ig hybridomas by SCRW. Viable HAT-selected hybridomas against the B7Ig fusion protein were encapsulated in biotinylated agarose microdrops. The agarose drops were 15–35 μm in size and approximately 7.5% of the drops were occupied. Under these conditions the

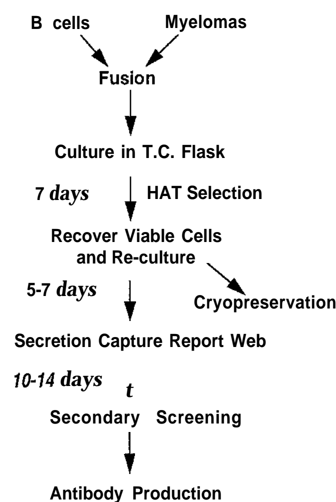

FIGURE 1. Flow diagram of the steps of MAb production using the Secretion Capture Report Web (SCRW).

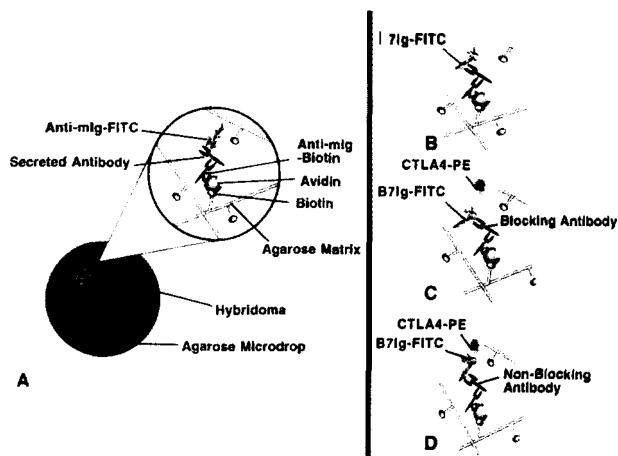

FIGURE 2. Design of the SCRW. Hybridomas are encapsulated in agarose microdroplets and incubated with capture and reporter reagents. The biotinyiated agarose matrix is depicted graphically in an expanded view. After cell encapsulation, the drops are incubated with avidin followed by biotinylated goat anti-mouse IgG. Antibody secreted by the antibody is thus captured within the matrix and then detected using fluorescent reporter(s): anti-mouse IgG-FITC (A), B7Ig-FITC (B), or B7Ig-FITC followed by CTLA4Ig-PE (C&D). The binding of reporters by blocking and non-blocking antibody is depicted in C and D, respectively.

TABLE 1. Comparison of the SCRW criteria and ELISA reactivities of SCRW-isolated hybridomas.

SCRW Region	SCRW Criteria	ELISA Reactivity (% Positive)* Ig secretors[b]	B7Ig specific[c]	B7 specific[d]	B7 specific blockers[d and e]
R1	Viable cells	96.8	52.4	40.8	1.6
R2	Mouse IgG+	100.0	73.5	44.4	7.4
R3	B7Ig+	100.0	96.6	66.9	12.6
R4	B7Ig high+	100.0	96.5	35.1	X.8
R5	B7Ig+, CTLA4Ig+	100.0	100.0	100.0	0.0
R6	B7Ig+, CTLA4Ig-	100.0	85.5	85.2	59.3

Supernatants were collected from 14 day cultures of hybridoma clones obtained from the regions described in Fig. 3 and analyzed as described in the Experimental Protocol.[a]Percentage of hybridomas per the total hybridomas from a SCRW region ($n \geq 58$) with: [b]≥20% binding activity in the mIgG ELISA,[c]≥20% binding activity in the B7Ig ELISA,[d]≥20% binding activity in the **B7Ig ELISA** and ≤20% binding activity in the CTLA4Ig ELISA,[e]≥50% blocking activity in the B7Ig.CTLA4Ig blocking ELISA.

probability is **97%** by Poisson statistics that occupied drops contain a single hybridoma. Multiply occupied drops are further excluded during flow cytometric analysis on the basis of forward- and side-light scatter'.

The drop suspension was successively incubated with avidin and biotinylated anti-mouse IgG to form capture sites within the drops (Fig. 2). Avidin is a multivalent ligand which binds biotin non-covalently and serves as a bridge between biotinylated agarose and the biotinylated anti-mouse IgG. The drops were incubated at 37'C for 30 minutes in culture media to enable antibody secretion by the hybridomas and subsequent capture by the anti-mouse IgG linked to the agarose. The drops were then labeled with one of several reporters; either (1) fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG, (2) FITC conjugated B7Ig or (3) FITC conjugated B7Ig followed by phycoerythrin (PE) conjugated CTLA4Ig. Labeling is typically peri-cellular[7] and the color and intensity of fluorescence, green for FITC and orange for PE, can be analyzed by flow cytometry.

Flow cytometric analysis of hybridomas examined with the SCRW is shown in Figure 3. Using FITC-conjugated anti-mouse Ig as a reporter we observed fluorescence intensities which varied nearly 100-fold, but with 97% of the hybridomas showing staining above control levels (Fig. 3, top panel). By contrast when encapsulated hybridomas were labeled with fluoresceinated antigen, i.e. B7Ig-FITC, only 17% of the HAT-selected cells showed a level of fluorescence above the control (Fig. 3, middle panel). Thus, the use of antigen-reporter to detect the secreted antibody apparently identified a subset of antigen-specific hybridomas among the larger population of IgG secreting hybridomas.

Two-parameter analysis of B7/Ig hybridomas by SCRW. When hybridomas in microdrops were labeled with B7Ig-FITC fusion protein followed by CTLA4Ig-PE fusion protein, the population of hybridomas detected by antigen could be further divided into two populations, one which fluoresced both green and orange and a rare population (-2%) which fluoresced only green (Fig. 3, bottom panel). Our interpretation of this result was that the population which fluoresced both green and orange was composed of hybridomas secreting antibody which bound the B7Ig fusion protein in a way that still allowed the binding of CTLA4Ig to B7Ig. Thus, these hybridomas would be expected to produce "non-blocking" antibodies. By contrast, the encapsulated hybridomas which fluoresced green and not orange after staining with both B7Ig-FITC and CTLA4Ig-PE were producing antibodies which bound B7Ig in a way that did not allow binding of CTLA4Ig. Thus, these hybridomas would be expected to produce "blocking" antibodies.

Evaluation of SCRW selected hybridoma clones. To test these interpretations of SCRW observations, encapsulated hybridomas labeled using the different reporters were cloned from selected regions described in Figure 3, Regions 1-6, by deposition of single drops into wells of 96-well plates. After treatment with agarase to release each sorted hybridoma from its agarose drop, the wells were cultured under standard conditions for two weeks. Supernatants were collected from the wells and tested in assays to detect secreted mouse IgG, antibody to B7Ig or CTLA4Ig and the ability to block the binding of the B7Ig to CTLA4Ig. The results of these assays for the different populations are presented in Table I.

Consistent with the flow cytometric results, >96% of the viable hybridomas (represented by R1, Fig. 3) were positive for antibody in their culture supernatant (R1, Table 1). Approximately 52% of the viable hybridomas secreted an antibody which was positive for B7Ig by ELISA. Thus a higher percentage of hybridomas is positive by ELISA than would have been predicted by the SCRW (17%). This difference may be due to different sensitivities of the ELISA and the SCRW or the use of fluoresceinated antigen in the SCRW. Of the antigen positive hybridomas, most recognized only the B7 portion of the B7Ig molecule (~41% of the total population). Thus, as measured by ELISA, almost all of the hybridomas were secreting antibody and half produced antibodies with good binding activity towards B7Ig. By contrast, only 1.6% of the viable hybridomas were positive for blocking antibody as defined by the ability to inhibit ≥50% the binding of B7Ig to CTLA4Ig.

All of the hybridomas selected by the SCRW as either mIgG+ (R2) or B7Ig+ (R3–6) were secreting antibody as measured by ELISA. B7Ig ELISA binding activity increased to 73.5% for the population of mouse IgG+ hybridomas (R2) and to 96.6–100% for B7Ig+ hybridomas (R3–6). The percentage of B7-specific hybridomas within the mIgG+ population was similar to the percentage in the viable hybridoma population (44.4 and 40.8%, respectively) and increased to 66.9–100% for the B7Ig+ populations R3, R5 and R6. For the B7Ig high+ population (R4), B7-specific activity was 35.1% suggesting a bias toward Ig binding within this population. Blocking anti-

body activity increased to 7.4% for mouse Ig+ hybridomas and to 12.5 and 8.8% for B7Ig+ and B7Ig high+ hybridomas, respectively.

Two parameter selection of hybridomas. The most striking separation of blocking activity was seen for the hybridomas selected by SCRW **with** B7Ig-FITC followed by CTLA4Ig-PE. As predicted, none of the hybridomas which were B7Ig+,CTLA4Ig+ by SCRW (R5) were positive for blocking antibody activity by ELISA. By contrast 59.3% of the hybridomas which were B7Ig+,CTLA4Ig– by SCRW (R6) were also positive for blocking antibody activity by ELISA. The percentage of hybridomas with blocking antibody activity in the B7Ig+,CTLA4Ig– selected population was 5- to 6-fold higher than the B7Ig+ only selected population and 37-fold higher than the total viable hybridoma population. Thus, the SCRW identified hybridomas secreting antibodies which bound to unique epitopes (i.e. blocking or non-blocking) and enriched for hybridomas with a rare (1.6%) phenotype (i.e., blockers).

Stability of SCRW selected hybridoma clones. Traditional methods using several cycles of screening, expansion and limit-dilution cloning commonly reveal unstable clonal phenotypes. Since the SCRW screens and clones individual hybridomas directly from a bulk culture only two weeks after the fusion, we were concerned that fewer cycles of cloning and expansion would result in a high frequency of unstable clones. Therefore, the stability of 12 clones, both blocking and non-blocking types, obtained by SCRW was studied by culturing the clones for an additional 4 weeks then subcloning them by flow cytometric single cell deposition cloning without further SCRW selection. After 2 weeks in culture the supernatants of the subclones were tested for B7Ig binding activity by ELISA. All subclones of 9 SCRW cloned hybridomas and >97% of subclones of the remaining 3 SCRW hybridoma clones were positive for B7Ig binding by ELISA (data not shown).

Discussion

The secretion capture report web has several features that are advantageous for MAb development These include: (1) Individual hybridoma cells are screened and cloned in a single step. (2) The method rapidly scans a single culture of all the hybridomas which together constitute a "library" of the entire array of antibodies. (3) This library can be expanded, cryopreserved and repeatedly sampled using SCRW assays with various configurations to isolate second and third generation monoclonal antibodies with novel properties.

Rare hybridomas can be identified and isolated with the SCRW given the high rate of analysis and sorting possible with flow cytometry. In these studies, less than one hour of flow cytometric analysis and sorting generated 64 clones which blocked the ELISA binding of B7 to CTLA4; an activity found in only 1.6% of the total viable cell population. Standard culture techniques would require many thousands of wells to reveal a similar population.

The SCRW format is amenable to a variety of analyses. By adapting the capture and reporter reagents, the SCRW can be configured to simultaneously analyze several characteristics of each hybridoma's monoclonal product including antigen specificity, epitope specificity, isotype and secretion level (the present report, ref. 7 and unpublished data). In addition to the studies reported here, the technique has been used to obtain rare blocking MAbs to CTLA4Ig, to clone hybridomas to a 34 aa peptide[7] and to isolate hyper-secretory hybridomas'.

Experimental Protocol

Immunization of mice, fusion and culture of hyhridomas. Mice were injected s.c. 3 times at 2 week intervals with 50 μg of B7Ig in either Alhydrogel/MDP or SAF-1 adjuvant prepared as previously describe&'. Two mice selected for fusion were given an i.v. injection of 100 μg of B7Ig at day 3 and 4 before the fusion. Splenocytes were fused with P3X63-Ag8.653 myeloma cells using 50% PEG (Boehringer-Mannheim, Indianapolis, IN). Cells were cultured at 5×10^6 cells/ml in two 225 cm^2 tissue culture flasks each containing 45 ml of HAT media: HB 101 media (Irvine Scientific, Irvine, CA), 7% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 X HAT, and 10% Hybridoma Cloning Factor (HCF; IGEN, Rockville, MD). Two days after fusion an additional 45 ml of HAT media was added. Four days after fusion the supernatant was removed from the flask, passed through a 70 μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ), centrifuged at 500×g for 5 min. and the pellet re-cultured in the same tissue culture flask with 45 ml of fresh HAT media, Seven days after the fusion adherent cells were removed using a cell scraper, the cells were recovered by centrifugation and viable cells were isolated using Lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada), Viable hybridoma cells were re-cultured in HAT media at 0.5-10×10^5 cells/ml.

Secretion **Capture Report** Weh. B7Ig and CTLA4Ig were produced as previously described'. B7Ig (0.27 mg) was conjugated with fluorescein isothiocyanate[10] and CTLA4Ig (0.47 mg) was conjugated to R-phycoerythrin[11] by Chromaprobe. Inc. Mountain View. CA. Fourteen days after fusion hybridomas were collected by centrifugation and viable cells isolated using Lympholyte M. Cells resuspended in 0.3 ml FBS were mixed with 0.3 ml of 4% biotinylated low-melt agarose (FMC Bioproducts, Rockland, ME), both at 37°C. to yield a 2% final agarose solution containing 4.5×10^6 cells. Hybridomas were encapsulated in agarose microdrops using a CellSys 100™ microdrop maker (One Cell Systems) as previously described'. Alternatively. agarose-encapsulated cells can be prepared by adding the cell suspension slowly, over about 1 minute, to 16 ml of 500cs dimethylpolysiloxane (Sigma, St. Louis, MO) stirred using a 1 inch stir bar in a sterile 30ml beaker and a magnetic stirrer at medium speed. Then, the mixture is stirred for 10 minutes at slow speed in an ice-water bath. The gelled microdrops were recovered from the oil by centrifugation for 11 min. at 1600×g of 7.5 ml of the suspension through 6 ml PBS underlayers in each of two 50 ml conical tubes. The drops were pooled and washed by centrifugation through 50 ml of PBS. The drops were incubated 5 min. at R.T. with avidin (2 mg in 4 ml PBS/1%FBS), washed twice with PBS/1%FBS and then incubated 5 min. at R.T. with biotinylated goat anti-mouse IgG (Zymed Labs. Inc., S. San Francisco, CA; 0.4 mg in 4 ml PBS/1%FBS). The drops were then added to 30ml of 37°C HT media (HB101 media. 7% FBS, 2 mM L-glutamine. 1 mM sodium pyruvate, 1X HT, and 10% HCF) and incubated with rocking for 30 minutes to allow secretion and capture of antibody. Then, the drops were washed twice with 50ml PBS, Approximately one-half of the drop suspension was incubated with 0.2 mg of goat anti-mIgG-FITC

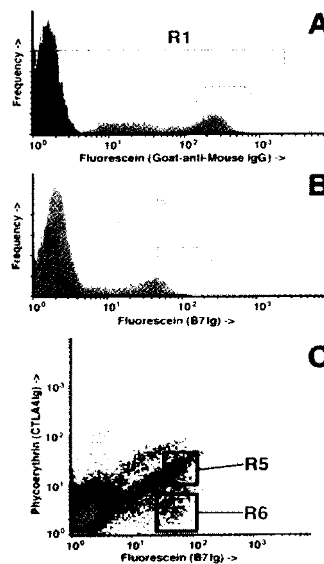

FIGURE 3. Flow cytometric analysis of the SCRW. Hybridomas raised to B7Ig were examined with the SCRW using either anti-mouse IgG-FITC (top panel), B7Ig-FITC (middle panel), or B7Ig-FITC followed by PE-CTLA4Ig (bottom panel). R1-6 designate regions from which specific populations of hybridomas were cloned and analyzed as described in Table 1. Background fluorescence was determined using encapsulated hybridomas in which the capture antibody, anti-mouse IgG biotin, was omitted from the staining procedure (shown in gray and gray outline).

(Zymed Labs) or 0.05 mg of B7Ig-FITC in 4ml PBS/1%FBS for 15 minutes on ice. One-half of the B7Ig-FITC labeled drop suspension was washed twice with 50 ml of PBS and incubated with 0.05 mg of CTLA4Ig-PE in 4 ml PBS/1%FBS for 15 minutes on ice. These conditions are sufficient to saturate the potential capture and reporter sites within the SCRW and minimize the diffusion of secreted antibody between drops. After a final wash with PBS, 5 μg/ml propidium iodide was added which fluorescently labels the non-viable cells and debris red. The preparation was filtered through a 70 μm cell strainer and analyzed on a FACStar Plus using a 100 μm orifice. Drops which contained more than one hybridoma or were non-viable were excluded from the analysis criteria by forward-light scatter and propidium iodide fluorescence, respectively. Hybridomas meeting analysis criteria were cloned using the Automatic Cell Deposition Unit into wells of a 96-well plate containing 200 μl of HT media with 4 units/ml of freshly prepared agarase (Sigma, St. Louis, MO). Cloning efficiencies in these experiments ranged from 2047% and efficiencies up to 65% have been obtained.

Solid-phase antigen ELISAs for secreted antibody. ELISAs were performed as previously described[12]. Ninety-six well ELISA plates were incubated with 50 μl/well of either 10 μg/ml goat anti-mIgG (Zymed Labs), B7Ig or CTLA4Ig in PBS overnight at 4°C. After blocking and washing the wells, 25 μl of hybridoma supernatant plus 25 μl of wash buffer were added to the wells and the plates incubated for 2 hours at room temperature. After washing the wells, 50 μl of a 1:2,000 dilution of goat anti-mouse IgG peroxidase conjugate (Zymed Labs) was added and the wells incubated for 1.5 hours at room temperature. After washing, the wells were incubated with o-phenylenediamine solution and the absorbance at 45Onm was measured. The absorbance obtained using supernatant from wells without hybridomas and B7Ig-immunized mouse sera (1/100 dil.) were used to set the values for 0% and 100% binding activity, respectively.

ELISA **for blocking antibody.** Biotin conjugated CTLA4Ig was prepared as previously described'?. Ninety six-well ELISA plates were incubated with 50 μl/well of 5 μg/ml of B7Ig in PBS overnight at 4°C. After blocking and washing the wells, 25 μl of supematant from the cloned hybridomas plus 25 μl of 1 μg/ml biotin conjugated CTLA4Ig was added to the wells and the plates incubated for 2 hours at room temperature. After washing the wells, 50 μl of a 1:3,000 dilution of streptavidin peroxidase conjugate (Zymed Labs) was added and the wells incubated for 1 hour at room temperature. After washing, the wells were incubated with o-phenylenediamine solution and the absorbance at 450nm was measured. The absorbance obtained using supematant from wells without hybridomas and the B7-1 blocking MAb BB1 (Becton Dickinson, San Jose, CA) were used to set the value for 0% and 100% blocking activity, respectively.

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