Preface

It is now widely accepted that the extracellular matrix (ECM) is a key determinant of tissue-specific gene expression. Signals provided by ECM are transduced by integrins, a large and growing superfamily of transmembrane heterodimeric cell surface receptors that link the ECM to structural and functional elements within the cell. A wide range of cellular phenotypes have been shown to be regulated by integrins, including growth, differentiation, migration, invasion, angiogenesis, and apoptosis. Furthermore, abnormalities of integrin expression and function have been implicated in the etiology of various pathologic conditions, including cardiovascular disease, inflammatory disorders, and cancer. Thus integrins have emerged as an important class of molecules with wide ranging implications for understanding basic biological processes.

In *Integrin Protocols* we provide a wide-ranging collection of laboratory protocols intended to assist investigators interested in integrins in working productively with these molecules, in studying their expression, and in potentially manipulating that expression to define their role(s) in relevant biological models. Protocols are provided for the analysis of integrin expression both at the RNA and protein levels (Chaps. 2, 5, and 7). Delcommenne and Streuli describe procedures for making rat monoclonal antibodies specific for mouse integrins; Schneller et al. and Arap and Huang describe methods for western blotting of integrins and RT-PCR analysis. Protocols are included that cover the analysis of the functional properties of integrins (Chaps. 1, 3, 4, 8, and 9 through 11). Koivunen et al. discuss methods for isolating integrin binding peptides from phage display libraries; Oktay et al. and Wary et al. describe protocols for assaying integrin-mediated kinase activity and shc signaling; Li and Arnaout describe the functional analysis of the β_2 integrins; Steger et al. discuss methods for analyzing the function of heterologous integrin genes; Edwards and Streuli provide methods for producing function blocking anti-integrin antibodies; and Talts et al. describe methods for knocking out integrin genes by homologous recombination.

The analysis of integrin function at the cellular level is described in Chapters 13 through 18. Wu et al. provide protocols for assessing and manipulating

integrin-mediated cellular adhesion; von der Mark and Goodman and Price and Thompson describe methods for studying cellular migration and invasion; Frisch discusses methods for the study of integrin-regulated epithelial cell apoptosis (anoikis); Brooks et al. address the role of integrins in angiogenesis and Somasiri and Roskelley describe protocols for assessing integrin signaling in the differentiation of mammary epithelial cells. Finally there is a chapter by Hirsch et al. on the analysis of the regulation of integrins themselves (Chap. 12).

Thus *Integrin Protocols* has been compiled from the viewpoint of understanding how integrins regulate normal and diseased cellular function and describes many of the currently available tools necessary to accomplish this task. The book should be useful to a broad range of investigators interested in this important class of molecules in both an academic and industrial setting.

Anthony Howlett

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Production of Rat Monoclonal Antibodies Specific for Mouse Integrins

Marc Delcommenne and Charles H. Streuli

1. Introduction

Monoclonal antibodies (MAbs) have proved to be an important tool for identifying novel cell-adhesion molecules and for determining their ligand binding specificity, function, and structure. Consequently, they have been instrumental in defining three families of adhesion receptors: the cadherin, immunoglobulin, and integrin families. Integrins include over 20 adhesion receptors that react with the extracellular matrix or cell-surface molecules. Integrins are composed of two distinct transmembrane glycoprotein subunits, α and β, which are noncovalently linked to each other. At least 15 α chains and 8 β chains have been observed in several possible combinations that determine the ligand specificity and function of the complex *(1)*.

To this date, MAbs have been produced against virtually all human integrin chains and most of them are now commercially available from several companies. However, MAbs specific for integrins of other species are still scarce and unquestionably needed. In addition, production of novel MAbs against human integrins remains important for epitope mapping *(2–4)* and dissecting integrin-mediated signal transduction pathways *(5)*. Finally, some applications require large amounts of MAbs and preparing home-made anti-integrin MAbs can be very cost effective. Therefore, we shall detail below the procedure we followed to produce and screen rat MAbs against mouse integrins that was successful in our hands and that can be adapted for specific purposes.

1.1. Strategy

Four factors are important for the successful generation of MAbs: the species of the animal used for immunization, the type of the antigen, the immunization scheme, and the screening procedure.

Mice are generally used for hybridoma generation but are seldom used for raising antibodies against mouse receptors because of their tolerance to most syngeneic antigens. Hamsters or more often, rats, are currently used instead. Rats are in easy supply and provide a high number of spleen lymphocytes available for fusion with myeloma cells. Moreover, hamster hybridomas grow slowly initially and were reported to sometimes be overgrown by fibroblast-like cells *(6)*.

Affinity-purified receptors, purified membranes, or cells have been used as immunizing material for anti-integrin MAb production. Immunization with purified receptors increases the probability of obtaining specific hybridomas. However this strategy necessitates obtaining the receptor in sufficient quantities for immunization, either by direct purification or by immunoaffinity purification on a first generation mono- or polyclonal antibody column. In addition, MAbs raised against purified integrins are frequently not function-blocking and are therefore of limited use. Immunization with purified membranes has been used successfully for hybridoma production *(7)* but this procedure requires additional material and we shall not describe this protocol here. We have chosen the injection of whole cells, which are highly immunogenic and are simple to obtain and use. Although this procedure presents a disadvantage in that hybridomas are produced against virtually all cellular antigens and require several screening steps to select for anti-integrin hybridomas, the immunization protocol is easy and function-blocking antibodies can be obtained. Choice of the cell type used for immunization is also particularly important in this respect since cell lines expressing high levels of the integrins of interest will increase the chance of generating hybridomas against these receptors. Cell types expressing immunodominant antigens such as mucins have to be avoided as they have been shown to hamper the immune response against other cell surface antigens (P. J. Kilshaw, pers. comm.).

Although immunization schedules can vary widely, most of them have been devised to promote both amplification of high-affinity antibodies producing B-cell clones and the availability of proliferating B cells on the fusion day, as only these cells can fuse with the myeloma. Thus, multiple injections of the antigen emulsified with adjuvant are performed; this constantly releases antigen into the circulation, promoting continuous proliferation of specific B cells. In addition, the fusion is performed 3 d after the final boost, when the activated B cells are still proliferating but not yet differentiated into secreting plasma cells.

The screening protocol has to be established prior to the fusion so that hybridomas reacting with the antigen of interest can be selected rapidly and amplified (*see* **Note 1**). In our procedure, MAbs were first selected by indirect immunofluorescence of cell-surface staining followed by flow cytometry. Next, we focused our attention to MAbs that immunoprecipitated heterodimeric

complexes showing two migrating bands of 90–110 and 140–180 kDa on nonreducing SDS-PAGE gels.

1.2. Outcome

To produce MAbs against mouse β_1 and α_v integrins, we selected the mouse melanoma K1735-M2 cell line as an immunogen because it expresses most β_1 integrins *(8)* and also $\alpha_v\beta_3$ at a high level. Spleen cells from rats immunized with this melanoma cell line generated a hybridoma according to the screening protocol described below, which was specific for mouse α_v integrin. This MAb, C8F12, is now being used to monitor the physiology and differentiation of various cell lineages *(9)*. This antibody is being used further to generate antisera against $\alpha_v\beta_3$ and second generation MAbs against mouse β_1, β_3, β_5, β_6 and β_8 chains as they all combine with the α_v subunit.

2. Materials

2.1. Animals and Cell Lines

1. Animals for immunization: 6-wk-old female Lou rats, purchased from OLAC (Shaw's Farm, Blackthorn, Bicester, UK) (*see* **Note 2**).
2. IR983F rat myeloma cells, deficient in hypoxanthine phosphoribosyltransferase (HPRT) and adenosine phosphoribosyltransferase (APRT) (*see* **Note 3**) *(10)*.
3. K-1735 M2 mouse melanoma cells. These cells are passaged at 1:10 or 1:20.
4. Human embryonic lung cells, MRC-5 (ATCC #CCL 171). These cells are passaged at 1:3, but should be used before passage 40 *(11)*.

2.2. Material for Routine Cell Culture

1. Cell culture medium for routine culture of MRC5, IR983F, and K-1735 M2 cells: Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Paisley, Scotland #12100-046) prepared according to the manufacturer's directions supplemented with 1 x non essential amino acids solution (Life Technologies cat. no. 11140-050), 10% fetal calf serum (FCS) (Life Technologies cat. no. 16000-044), solution of 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies cat. no. 15140-122), 50 µg/mL gentamycin (Life Technologies cat. no. 15750-029), 3.7 g/L sodium bicarbonate (Sigma, St. Louis, MO, cat. no. S 5761). Media supplements are stored as concentrated stocks at –20°C, and media are stored at 4°C.
2. Phosphate-buffered saline (PBS): to make 1 L of 10× stock solution, dissolve 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4, and 11.25 g of Na_2HPO_4, adjust to pH 7.4, and bring to a total volume of 1 L. Autoclave the solution of 1× PBS at 121°C for 20 min and store it at room temperature.
3. PBS–EDTA 0.2%: to make 1 L, dissolve 2 g of EDTA in 800 mL distilled water and adjust the pH to 8.0 with 5 *M* NaOH to help dissolve EDTA. Then add 100 mL of 10× PBS, adjust the pH to 7.4, and bring to a total volume to 1 L. Sterilize by autoclaving at 121°C (15 psi) for 20 min, and keep at room temperature.

4. 0.25% Trypsin, 1 m*M* EDTA solution (Life Technologies cat. no. 25200-056). Store in aliquots at –20°C.
5. Cell storage solution: Mix 5 mL of DMSO (Sigma cat. no. D 8779) with 10 mL of FCS and 35mL of DMEM and keep at 4°C.
6. 100-, 250-, and 500-mL glass bottles for medium.
7. Sterile 5-, 10-, and 25-mL glass pipets.
8. Multichannel pipet.
9. 50-, 100-, and 150-mm tissue-culture dishes (Nunc, Roskilde, DK).
10. 25- and 75-cm^2 tissue-culture flasks (Falcon, Los Angeles, CA).
11. 1.5-mL sterile Eppendorf tubes.
12. Sterile 15- and 50-mL centrifuge tubes (Falcon).
13. Refrigerated bench-top centrifuge (with holders for 15, 50-mL tubes and 96-well microplates).

2.3. Material for Immunization and Boosts

1. Complete Freund adjuvant for the first injection (Sigma cat. no. F 5881) (*see* **Note 4**).
2. Incomplete Freund adjuvant for the boosts (Sigma #F 5506).
3. 1-mL syringes.
4. 21-gage needles.

2.4. Material for Feeder Layer Preparation and Cell Fusion

1. Mitomycin C solution: Dissolve mitomycin C (Sigma cat. no. M 0503) at 1 mg/mL in H$_2$O, filter sterilize, and store at 4°C. This solution is stable for at least 4 mo.
2. Hybridoma culture medium (H medium): To 500 mL of cell culture medium described above (DMEM, 10% FCS), add 50 mL of horse serum (Life Technologies cat. no. 16050-122) and 5.5 mL of hypoxanthine 100× stock solution. This stock solution is prepared by dissolving 136 mg of hypoxanthine (Sigma cat. no. H 9636) in 100-mL H$_2$O heated to 70°C. The 100× stock solution is filter sterilized, aliquoted, and stored at –20°C.
3. Hybridoma selection medium (HA medium): To 250-mL hybridoma culture medium (H medium), add 250 µL of 1000× stock solution of azaserine. This solution is prepared by dissolving 10 mg of azaserine (Sigma cat. no. A 1164) in 10 mL of H$_2$O. The azaserine 1000× stock solution is filter sterilized, aliquoted, and stored at –20°C (*see* **Note 5**).
4. Spleen cell resuspension medium: DMEM containing 2.5% FCS and antibiotics (*see* **Subheading 2.2.1.**) kept at 4°C.
5. Polyethylene glycol (PEG): 50% solution in PBS (Sigma cat. no. P7181).
6. 1-L and 200-mL beakers.
7. 500 mL of 70% ethanol.
8. Dissection board.
9. Medium-sized scissors, fine scissors, and fine forceps autoclaved for 20 min at 121°C.
10. Sterile 21-, and 25-gage needles.
11. Sterile 20-mL syringes.
12. Water bath at 40°C.
13. Timer.

14. Sterile 1-mL glass disposable pipets (Volac, John Poulten Ltd, Barking, UK).
15. Sterile 96-well flat-bottomed plates (Nunc cat. no. 1-67008A).
16. Sterile 24-well flat-bottomed plates (Nunc cat. no. 1-43982).

2.5. Additional Material for Screening

1. 20% NaN_3 stock solution: dissolve 20 g of NaN_3 (Sigma cat. no. S 2002) in 100 mL of H_2O.
2. 96 conical-well microplates (Nunc cat. no. 442587).
3. FITC-conjugated Rabbit anti-rat IgG antiserum (Sigma cat no. F 1763).
4. PBS–2% formaldehyde: Dilute 5 mL of formaldehyde 40% (Merck, Rahway, NJ, cat. no. B10113-76) in 95 mL of PBS and keep at 4°C.
5. Flow cytometer (FACSscan, Becton Dickinson, Rutherford, NJ).
6. [^{35}S]-Methionine-labeling reagent: Tran^{35}S-label (ICN Biomedicals, Thame, UK cat. no. 51006) at 10 mCi/mL.
7. Low-methionine DMEM: Prepare this medium by adding 3.5 g of glucose and 3.7 g of sodium bicarbonate to 1 L of cystine–methionine-deficient, low-glucose DMEM (Sigma cat. no. D3916) and filter sterilize it. Mix 900 mL of this medium with 100 mL of the regular DMEM to obtain the low-methionine medium.
8. Dialyzed FCS: Sterilize a dialysis membrane by autoclaving at 121°C for 20 min. Pour 20 mL of FCS into this membrane in a laminar flow hood and dialyze it at 4°C against 3× 1 L of autoclaved 150 mM NaCl saline solution. Filter sterilize, aliquot the dialyzed FCS, and store at –20°C.
9. Lysis buffer: This buffer contains 150 mM NaCl, 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP_40, pH 7.4, and is supplemented just prior to use with the protease inhibitors 1 mM PMSF (Sigma #P 7626), 0.2 trypsin inhibitor units (TIU)/mL aprotinin (Sigma #A 6279), 10 µg/mL leupeptin (Sigma #L 2023), 10 µg/mL pepstatin (Sigma #P 4265). Keep this buffer at 4°C.
10. Activated charcoal (Sigma cat. no. C 5260).
11. Protein G Sepharose beads (Zymed Labs Inc., South San Francisco, CA, cat. no. 10-1242).
12. [^{14}C]-labeled molecular weight markers (Amersham cat. no. CFA 756).
13. 30% solution of trichloroacetic acid, stored at 4°C.
14. GF/C glass microfiber filters (Whatman International, Maidstone, UK).
15. Scintillation fluid.
16. Scintillation counter.
17. Fluorographic reagent: Amplify, (Amersham, Arlington Heights, IL cat. no. NAMP 100).

3. Methods

3.1. Protocol of Rat Immunization with K1735-M2 Cells

1. Plate K1735-M2 cells on 150-mm dishes at 10^6 cells/dish and grow until subconfluent.
2. To detach the cells, wash the monolayers with PBS–EDTA, and harvest the cells by incubating them with 5 mL of PBS–EDTA per 150-mm dish for 5–10 min at 37°C.

3. Transfer the pooled detached cells to a 50-mL tube and wash them three times with serum-free DMEM.
4. Resuspend 20×10^6 cells in 250 µL of PBS and aspirate them in a 1-mL syringe.
5. Transfer 250 µL of complete Freund adjuvant for the first injection to an Eppendorf tube.
6. Mix cell suspension with adjuvant and then emulsify by passing the mixture rapidly back and forth between the syringe, fitted with a 12-gage needle, and the Eppendorf tube.
7. Remove some blood from each rat before the first injection to have a preimmune control antiserum. Then prime the rats by multiple subcutaneous injections.
8. Boost the rats 2–3 times at monthly intervals by injecting intraperitoneally 20×10^6 cells emulsified in Freund incomplete adjuvant.
9. Boosting should continue until the antiserum titer, assessed by immunofluorescence, is at least $1/10^4$ (*see* **Subheading 3.3.1.**). Remove approx 200 µL of blood from rat by tail bleed. After letting the clot form by incubation for 1 h at 37°C, recover the antiserum from the clot and spin it at 1500g in a minifuge to discard residual cells. Store the aliquots of antiserum at –20°C.
10. For the final boost, inject 20×10^6 cells resuspended in 500 µL of PBS intraperitoneally 3–4 days prior to fusion.

3.2. Fusion Protocol

3.2.1. Preparation of MRC5 Cells Feeder Layer

The feeder layer is prepared 3 d before fusion. This period allows the medium to be conditioned for optimal survival and growth of the hybridomas.

1. Three days before cell fusion, discard the medium from exponentially growing MRC5 cells in 150-mm dish and incubate the cells for 1 h with 15 mL of fresh culture medium plus 30 µL of mitomycin C stock solution (*see* **Note 6**).
2. Wash the cells 3× with DMEM serum-free medium, 1× with PBS–EDTA, and then detach them with 2 mL of trypsin-EDTA.
3. Resuspend MRC5 cells in hybridoma selection medium (HA medium) at the density of 8×10^4 cells/mL.
4. Distribute 100-µL aliquots in a 96-microwell plate using a multichannel pipet (*see* **Note 7**).

3.2.2. Preparation of Myeloma Cells

1. Maintain the IR983F myeloma cells in strict exponential growth for at least 1 wk before fusion to maximize their fusion capacity. To maintain exponential growth, adjust the cell density to 0.5×10^5 cells/mL (i.e., 1.5×10^6 cells/30 mL in 150-mm dish) 2, 4, and 6 d before fusion.
2. Because IR983F cells are rather adherent, detach them with PBS–EDTA 0.2%, transfer the cell suspension to a 50-mL tube, and fill the rest of the tube with DMEM.

3.2.3. Preparation of Spleen Cells

1. Euthanize the rat by cervical dislocation and then dip it in a 1-L beaker filled with 500 mL of 70% ethanol.
2. Place the rat in a sterile laminar flow hood on its right hand side on a dissection board which has been cleaned with 70% ethanol.
3. Pinch up the superficial skin over the left side of the abdomen and make a 5-cm incision over the spleen. Then dissect the skin from the muscular wall and deflect the edges laterally to expose the abdominal wall through which the spleen is visible.
4. Using sterile fine forceps, incise the muscle layers and peritoneum over the spleen with fine scissors. Then, exteriorize the spleen by gently lifting its lower pole.
5. Release the spleen by cutting its mesentery and transfer it to a 50-mm Petri dish containing approx 5 mL of ice-cold DMEM–2.5% FCS.
6. After rinsing, transfer the spleen to a fresh 100-mm Petri dish and dislodge the spleen cells using two 20-mL syringes fitted with 26-gage needles, each containing 20 mL of DMEM–2.5% FCS. To achieve this, one syringe is used to anchor the spleen, while the other is used to flush fluid into successive small areas of spleen.
7. Transfer the cell suspension to a 50-mL tube leaving behind the spleen carcass and small lumps of tissue that may have settled on the base of the dish. Allow the remaining clumps and pieces of connective tissue to sediment for approx 5 min, then transfer the cell suspension to another 50-mL plastic tube.
8. Spin the tube at room temperature for 10 min at 400g.
9. Resuspend pellet in 10 mL of serum-free DMEM medium. To count the cells, take a 100-µL aliquot of the cell suspension and mix it with 810 µL of distilled water by gentle shaking for 30 s to lyse the erythrocytes, then add 90 µL of 10× PBS to restore the physiological osmolarity. The cell viability must be at least 95% as measured by Trypan blue exclusion.

3.2.4. Fusion (**ref. 12**)

1. Mix 10^8 spleen cells with 2×10^7 IR983F myeloma cells in a 50-mL tube (5 spleen cells/1 myeloma cell). Add DMEM to a volume of 50 mL.
2. Spin the cells at room temperature for 8 min at 400g.
3. Aspirate the supernatant with a Pasteur pipet connected to vacuum. Make sure to remove the supernatant completely so as not to dilute PEG in the next steps.
4. Loosen the cells in the pellet by gently tapping the bottom of the tube. Place the tube in a 200-mL beaker containing water at 40°C and keep it there during the fusion.
5. Add 0.8 mL of 50% PEG prewarmed at 40°C to the pellet slowly over a period of 1 min using a 1-mL pipet, continuously stirring the cells with the pipet tip.
6. Continue stirring the cells in 50% PEG for a further 1.5–2 min.
7. With the same pipet, add 1 mL of DMEM, taken from a 50-mL tube containing 30 mL of DMEM kept at 37°C in another beaker at 40°C, to the fusion mixture, continuously stirring as before, over a period of 1 min.
8. Repeat **step 7**.

9. Repeat **step 7** twice, but add the medium in 30 s.
10. Always with the same pipet and continuously stirring, add 6 mL of DMEM over a period of approx 2 min.
11. With a 10-mL pipet add 12–13 mL of DMEM dropwise.
12. Spin down the aggregates at 400g for 5 min.
13. Discard the supernatant, break the pellet by gently tapping the tube, and resuspend the cells in hybridoma selection medium (H medium).
14. Adjust the cell density to 10^6 cells/mL and distribute 100 µL in each microwell of 96-well microplates containing MRC5 cells with a multichannel pipet.

3.2.5. Feeding Hybridomas

The medium is changed first on the fourth day after fusion. At this stage, all the nonfused myeloma cells are dead. Therefore, azaserine is no longer necessary and the cells are switched to hypoxanthine-containing culture medium (H medium). Half of the medium is removed from each microwell by suction using a Pasteur pipet connected to a vacuum and fresh medium is dispensed by using a multichannel pipet. Subsequently, medium is changed every 2 d, gradually diluting out residual azaserine and antibodies secreted by the unfused lymphocytes. Hybridomas will become apparent over the next 7–10 d, and should be screened before they become confluent (*see* **Note 8**).

3.3. Screening

The screening of supernatants generally begins 7–10 d after fusion, from hybridomas that cover a significant area of the microwell (at least one-fourth of the well area) and that have been refed at least three times.

3.3.1. Primary Screening of MAbs by Fluorescence Staining of M2 Cells

1. Detach K1735-M2 cells by PBS–EDTA treatment as described earlier, resuspend them at 20×10^6/mL in PBS–NaN$_3$ 0.1%, and distribute them as 50-µL aliquots to 96-v-well microplates.
2. Dispense 50 µL of hybridoma supernatant to each microwell and incubate them with the cells for 1 h on ice.
3. Wash the cells by dispensing 100 µL of cold PBS 0.1% and centrifuging the microplates at 400g for 5 min at 4°C.
4. Aspirate the supernatants with a Pasteur pipet connected to a vacuum line.
5. Resuspend the cells in 200 µL of PBS and pellet them again by centrifugation at 400g.
6. Resuspend the cells in 50 µL of FITC-conjugated rabbit anti-rat IgG antiserum diluted 1/100 in DMEM, 10% FCS, and incubate them for 1 h on ice.
7. Add 100 µL of PBS to the cells and spin the microplates at 400g for 5 min at 4°C.
8. Resuspend the cells in 100 µL of cold PBS and then dilute them in 100 µL of PBS–2% paraformaldehyde.
9. Analyze the cells by flow cytometry or fluorescence microscopy and keep the positive supernatants for the secondary screening.

3.3.2. Secondary Screening by Immunoprecipitation

1. Plate 10^5 K1735-M2 cells in a 50-mm dish.
2. When the cells have reached 70% confluence, wash them three times with sterile, low-methionine DMEM.
3. Feed the cells with 2 mL of low-methionine DMEM,10% dialyzed FCS, and 200 µCi/mL of Tran^{35}S-label.
4. Place the 50-mm dish in a box with two other 50-mm dishes, one containing activated charcoal to adsorb the volatile [^{35}S]sulfur, and a second one filled with distilled water, to keep the atmosphere water saturated.
5. Transfer the culture dish-containing box to a cell incubator at 37°C.
6. After an overnight [^{35}S]-methionine incorporation, wash the cells three times with warm serum-free medium, lyse the cells on ice with 0.5 mL of lysis buffer at 4°C.
7. Scrape the lyzed cells and transfer the lysate to an Eppendorf tube. Repeat once.
8. Vortex the pooled lysates and leave them on ice for 30 min. Then pellet nuclei and insoluble material by a 15-min centrifugation at 21,000g in a minifuge at 4°C. Aliquot the lysate supernatant and store it at –80°C until use.
9. Assess the level of [^{35}S]-methionine incorporated in proteins by trichloroacetic acid (TCA) precipitation on glass filters. Transfer the filters to scintillation vials, add scintillation fluid, and count the cpm in a scintillation counter.
10. For each supernatant to test, dilute in an Eppendorf a volume of lysate corresponding to 5×10^6 cpm with lysis buffer to reach a final volume of 200–500 µL. Then react 20 µL of hybridoma supernatant with the lysate for 1 h at 4°C under gentle shaking or rotation.
11. Add 25 µL of 50% (v/v) Protein G Sepharose in lysis buffer to each eppendorf and rotate the samples for 1 h at 4°C.
12. Pellet the beads by a 21,000g pulse in the minifuge and wash them three times with the lysis buffer and once with 50 mM Tris-HCl at pH 7.4.
13. Elute the immunoprecipitated antigens from the beads by boiling them for 3 min in 30 µL of nonreducing 2× sample buffer.
14. Run the samples along side [^{14}C]-labeled molecular weight markers on a nonreducing 6% SDS-PAGE gel.
15. Fix the gel in a solution of 40% H_2O, 50% methanol, 10% acetic acid, for 30 min, wash the gel in distilled water for 30 min and incubate the gel with Amplify.
16. Dry the gel.
17. Expose the gel to autoradiographic film or phosphorimager plates (**Fig. 1**).

3.3.3. Subsequent Screening Tests

After selecting a MAb immunoprecipitating a dimeric molecular complex similar to integrins, it is necessary to confirm specificity of the antibody. An initial step is to deplete the integrin from the lysate by repeated immunoprecipitations with a polyclonal antiserum reacting with the α or β subunit cytoplasmic domain of the suspected integrin and then to proceed with the MAb
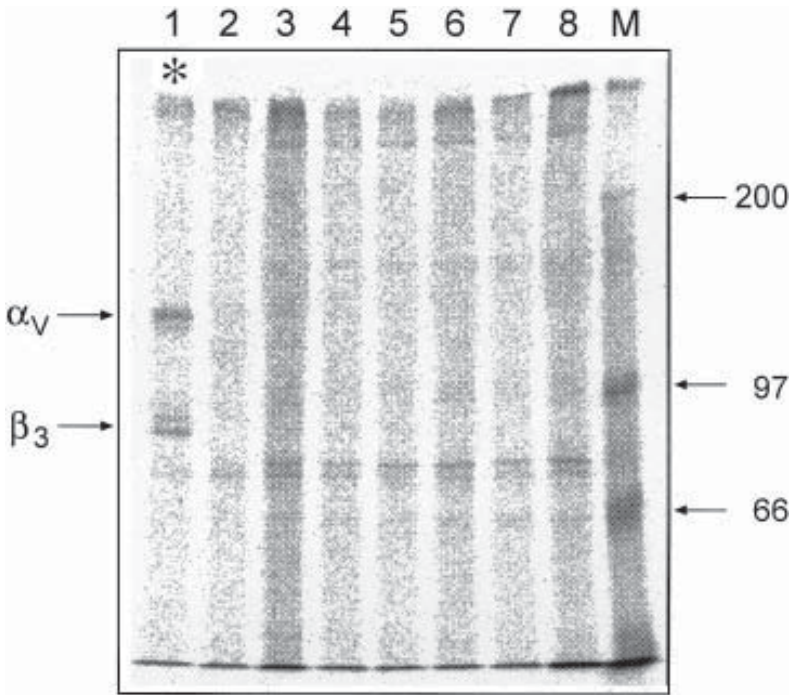
Fig. 1. Result of one initial screen to identify anti-integrin antibody. Hybridoma supernatants identified as positive in the primary fluorescence screen were tested for their ability to immunoprecipitate proteins with the approximate molecular mass of integrins. 5×10^6 cpm of a cell lysate of [^{35}S]-methionine-labelled K1735-M2 cells was immune precipitated with supernatants from eight hybridomas (lanes 1–8) and separated by SDS-PAGE. Sizes (in kDa) of the labeled molecular mass markers (Amersham cat. no. CFA 626) are shown in the margin. Only one supernatant-precipitated bands of the expected size (lane 1, indicated with an asterix). The antibody, MAb C8F12, subsequently turned out to recognize α_v integrin, and in this experiment β_3 integrin was coprecipitated.

(*see* **Note 9**). If this MAb no longer immunoprecipitates any material after immunodepletion, this will indicate that it reacts effectively with an integrin. The reverse experiment can also be performed: immunodepletion of the putative integrin with the MAb followed by immunoprecipitation with the polyclonal antiserum. The next step will be to ascertain the reactivity of the MAb with either one integrin subunit or an epitope formed by both α and β subunits. Determining the MAb specificity by Western blot should be attempted first. If the MAb does not work in Western blot, another method consists of performing immunoprecipitation after dissociation of the integrin heterodimer by increasing the pH *(13)* or SDS treatment *(14)*. Concurrently,

the MAb should be tested for immunoprecipitation using different cell lines expressing various integrins. If the MAb appears to immunoprecipitate different integrin dimers of the same subfamily, this will indicate that this MAb recognizes a common subunit (**Fig. 2**). Tissue-section immunocytochemical staining of different organs is also a good complementary test, particularly for MAbs recognizing integrins expressed in restricted tissues such as $\alpha4\beta_7$ *(15)*.

Finally, an important part of the MAb characterization consists of assessing its capacity to block cell adhesion to the extracellular protein(s) interacting with the suspected integrin. This can be done by quantifying the percentage of adherent cells after antibody treatment using an ELISA reader *(16,17)*.

3.4. Maintenance of Hybridomas

3.4.1. Expanding the Culture

During propagation of the positive hybridomas, it is advised to store a couple of samples of each in liquid nitrogen as soon as possible to have back-up cells should a contamination occur (*see* **Note 10**). Hybridomas are expanded as follows:

1. When the positive hybridomas cover at least 50% of the microwell area, 150 µL of medium are pipetted up and down to detach the cells. The cell suspension is then transferred to a 24-well dish containing 300 µL of H medium. Hybridoma cells left in the microwell are refed with 150 µL of H medium.
2. One day after the transfer, 1 mL of H medium is added if the cells are growing actively. Otherwise, this should be delayed.
3. When the cells cover the whole area of a 24-well dish, the medium is pipetted up and down to detach the cells and 1 mL of the cell suspension is transferred to a 25-mL flask already containing 3 mL of H medium.
4. One day later, 5 mL of fresh H medium are added to the cells.

3.4.2. Cell Storage

1. When the hybridomas are close to confluence, the 25-mL flask is shaken and the content is transferred to a 15-mL conical tube. An aliquot is taken and the cells are counted while the tube is spinning for 10 min at 400*g* (or 1500 rpm).
2. Cells are resuspended at $1–2 \times 10^7$ cells/mL in DMEM–10% FCS and mixed with an equal volume of DMSO storage solution, transferred to a –80°C freezer in a styrofoam box, and frozen to –80°C, then stored in liquid nitrogen.

3.4.3. Recovering Cells from Liquid Nitrogen Storage

1. Thaw the frozen cells by dipping the cryovial in a 37°C water bath.
2. Transfer the cell to a 50-mL tube containing 30 mL of warm DMEM, and spin the tube for 10 min at 400*g*.
3. Resuspend hybridoma cells into 1 mL of H medium and dispense them in a well of a 24-well dish. If many cells are viable, the medium will quickly become acid. Therefore, the cells will be transferred to a 25-mL flask. If cells are not numerous
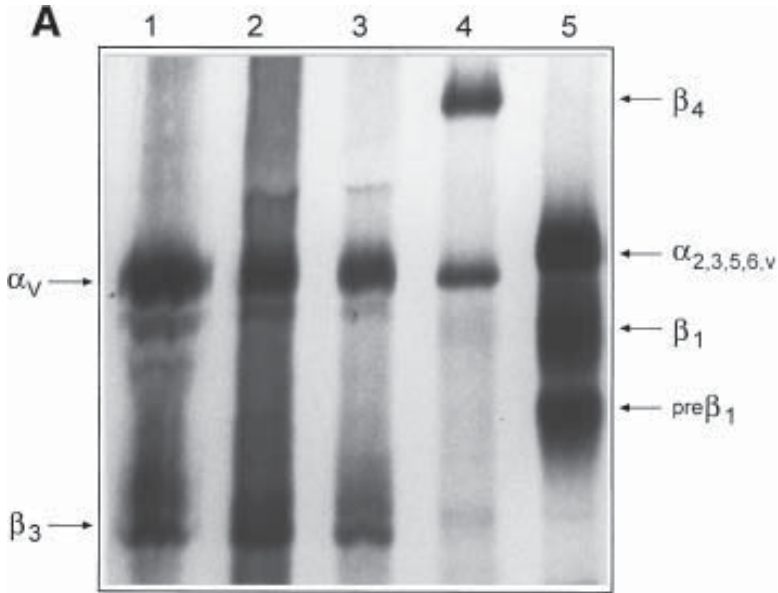
Fig. 2. MAb C8F12 recognizes α_v integrin. **(A)** Comparison of mouse integrin electrophoretic mobility with material immunoprecipitated by MAb C8F12. Primary mouse mammary epithelial cells were cultured overnight with [^{35}S]-methionine, after which they were lysed and subjected to immunoprecipitation with a rabbit anti-α_v cytoplasmic domain antiserum (lane 1), anti-mouse β_3 monoclonal antibody (lane 2), MAbC8F12 (lane 3), anti-mouse α_6 monoclonal antibody, GoH3 (lane 4), rabbit anti-β_1 cytoplasmic domain antiserum (lane 5). The immunoprecipitates were separated by SDS-PAGE on nonreducing 6% gels.

or healthy at thawing, it is safer to resuspend them to 1 mL of a mixture of MRC-5 cell conditioned medium diluted 1/4 in H medium.

3.4.4. Subcloning Hybridomas

After completion of hybridoma screening steps, it is necessary to ensure the monoclonality of the selected hybridoma. For this purpose, the hybridoma needs to be subcloned at least twice. Of the two methods currently in use, soft agar and limiting dilution cloning, we have adopted the latter for its simplicity.

1. Serially dilute the cells to a final density of 1 cell/mL.
2. Dispense cells as 100 µL aliquots in 96 well plates on a bed of mitomycin C–treated MRC5 cells (*see* **Subheading 3.2.4.**).
3. After the clones have grown sufficiently, test their supernatants by immunofluorescence and immunoprecipitation (*see* **Subheadings 3.3.1.** and **3.3.2.**).
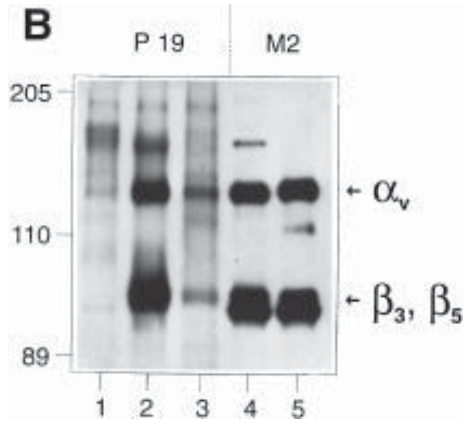4. Expand and clone positive clones a second time.

Fig. 2. *(continued)* MAb C8F12 recognizes α_v integrin. **(B)** MAb C8F12 was shown to recognize the mouse α_v integrin subunit by immunoprecipitation of α_v integrins from nondifferentiated mouse embryonal carcinoma P19 cells and K-1735 M2 cells. P19 cells express $\alpha_v\beta_5$ integrin but not $\alpha_v\beta_3$, whereas the reverse is true for K-1735 M2 cells. Lysates of cell surface biotinylated nondifferentiated P19 cells were reacted with anti-β_3 monoclonal antibody (lane 1), MAb C8F12 (lane 2) and rabbit anti-β_5 cytoplasmic domain antiserum (lane 3). Lysates of the cell-surface biotinylated M2 cells were reacted with MAb C8F12 (lane 4) or with anti-mouse β_3 monoclonal antibody (lane 5). Immunoprecipitates were separated on a 6% SDS-PAGE gel and transferred to PVDF membrane. This was incubated with streptavidin peroxidase and the cell-surface immunoprecipitated proteins were detected by ECL (Amersham) *(15,18)*.

5. It is advisable to reclone the hybridomas from time to time to eliminate the nonsecretory variants that may appear and overgrow the antibody secretory cells.

4. Notes

1. Originally, the first anti-integrin MAbs were screened according to their capacity to inhibit cell adhesion to extracellular matrix molecules *(19)*, cell–cell adhesion in immune response processes *(20)*, phagocytosis *(21)*, and clot formation *(22)*. We chose not to follow this strategy considering that it would lead to the loss of valuable nonfunction-blocking anti-integrin MAbs.

2. Although any rat strain can be used for immunization and fusion with the IR983F myeloma, which is of LOU origin, it is more convenient to use compatible LOU rats for injection so that the resulting hybridomas can be used to generate ascites tumor, if necessary.

3. Mouse myelomas can be used conveniently instead of IR983F cells for fusions with rat lymphocytes and several rat anti-integrin MAbs have been produced this way *(23,24)*. However, it has been claimed that rat hybridomas resulting from heterofusions happen to be unstable *(25)*.

4. Alternative adjuvants are acceptable, for example Titre-Max Gold.

5. Hybrids are commonly selected in a medium supplemented with HAT. Since IR983F cells are also deficient for adenine phosphoribosyltransferase, we preferred to use HA medium, principally for three reasons. First, selection with azaserine generates more hybridomas per fusion because loss of hybridomas with X chromosome segregation caused by culture in HAT medium does not happen in HA medium. Second, the proportion of IgG secreting hybridomas is higher when selected with azaserine as it eliminates hybridomas that are incapable of IgG heavy chain synthesis *(26)*. Thirdly, in case of hybridoma contamination by mycoplasmas, these microorganisms deplete thymidine from HAT-supplemented medium, causing the death of the hybridomas. However, hybridomas grown in HA medium will survive despite this contamination and can be treated with a suitable antimycoplasma drug.

6. Conventionally mouse peritoneal cells are used as feeder layer for hybridoma culture but are sometimes contaminated with pathogens. To minimize contamination risks, various cell lines have been used instead with mixed success *(27,28)*. Among the cell lines tested, MRC5 cells have been shown to provide an excellent feeder layer for hybridoma development partially because they secrete interleukin 6 *(29)*. Contrarily to what is stated in some methods books, it is crucial that MRC5 cells are treated with mitomycin C.

7. It is a good idea to set up 10× 96-well plates at this stage.

8. In a good fusion you might expect up to 60% of the wells to be showing signs of cell growth.

9. Antisera reacting with α and β chain cytoplasmic domains, available from Chemicon or Pharmingen can be used in these experiments. Because of the high conservation of sequence within the cytoplasmic domain, anticytoplasmic domain antibodies cross–react with integrins of most species. Incubation times, volumes of antibody to add to the lysate, and the number of sequential immunoprecipitations will depend on the antibody concentration and avidity and the level of integrin expression. Therefore, the immunodepletion protocol will have to be adapted for each case.

10. To avoid unnecessarily filling up a liquid nitrogen container, it is strongly recommended to discard frozen aliquots of hybridomas that are subsequently shown NOT to secrete useful antibodies.

Acknowledgments

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