[11] Quantitation of Hybridoma Immunoglobulins and Selection of Light-Chain Loss Variants

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Compared to conventional antisera, hybridoma antibodies offer many advantages including specificity, consistency, availability in large quantities, and the ability to use impure immunogens.[1] Hybridoma antibodies also differ in two other respects from conventional antibodies. First, some hybridomas secrete myeloma as well as specific antibody chains. Methods are described in Section I for selecting variant clones from mouse–rat or mouse–mouse hybrids that secrete homogeneous immunoglobulins. Second, in addition to measuring antibody activity, it is often desired to measure monoclonal antibody immunoglobulin concentration. The specific antibody component of monoclonal antibodies consists of a single heavy-chain subclass and light-chain isotype. Monoclonal antibodies therefore express only a portion of the antigenic determinants found in whole immunoglobulins. This has important implications for the measurement of monoclonal immunoglobulin concentration by immunoassay. Section II describes methods for measuring rat or mouse monoclonal immunoglobulins derived from rat–mouse, mouse–mouse, or rat–rat hybrids.

I. Quantitation of Myeloma Light-Chain Secretion and Selection of Loss Variants Using Radioimmunoassay

In immunoglobulin-synthesizing cells, the genes for immunoglobulin heavy and light chains are expressed by one chromosome each, and the expression of allelic genes on the homologous chromosomes are excluded. In hybridoma cells, the active genes for immunoglobulin synthesis from both the myeloma and spleen cell parents continue to be expressed.[1,2] Thus, two different heavy chains and two different light chains may be made by a single hybridoma cell. Theoretically, there are nine different combinations in which these chains can be assembled into an IgG. For purposes of nomenclature, the heavy and light chains from the spleen cell parent have been designated H and L, and those from the myeloma parent have been designated G and K, respectively. In HGLK

[1] G. Galfrè and C. Milstein, this series, Vol. 73, p. 3.
[2] C. Milstein, K. Adetugbo, N. J. Cowan, G. Köhler, D. S. Secher, and C. D. Wilde, *Cold Spring Harbor Symp. Quant. Biol.* **41**, 793 (1977).

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lines, i.e., hybrids secreting all four chains, hybrid molecules are assembled and secreted in which all permutations of heavy–light chain combinations occur and random heavy–heavy associations also occur unless the heavy chains differ in class.[3] If all chains are synthesized in equal amounts, then the bivalently active, specific, H_2L_2 antibody accounts for only 1/16th of the total antibody.

There are two approaches to obtaining more homogeneous and active antibody. First, variant myeloma lines are available as fusing partners that synthesize only the myeloma K chain or no chains at all.[1,4] The use of nonproducing myelomas is by far the easiest way of obtaining new hybridomas secreting homogeneous H_2L_2 antibodies. Second, from hybridomas previously prepared with myeloma-chain producing myelomas, such as P3-X63, NSI, or MPC 11, variant hybridomas may be selected that no longer secrete myeloma chains. There is also one point in favor of making new hybridomas with myeloma lines, such as NSI, that make a myeloma K chain. From an HLK hybrid, HK variants as well as HL variants can be selected. The HK antibodies serve as the best possible control for the HL antibodies, since they have exactly the same heavy chain, but in association with an inappropriate light chain that in almost all cases leads to loss of specific antigen-binding capacity. Such variants are also useful for studies on idiotypes and the antigen binding site.

Hybridoma lines lose specific and myeloma chains at similar frequencies.[5] The pattern of chain loss is essentially random, except that hybridomas with an excess of expressed heavy chains over light chains are rarely seen.[5] This may be due to toxicity by free heavy chains. Loss of chains is correlated with loss of specific chromosomes, 6 and 12 for mouse kappa and heavy chains, respectively.[6] When mass cultures are screened for variants, they are usually found at frequencies of 1/50 to 1/200.[7,57]

The effects of various chain loss events in NSI × spleen cell HLK hybrids are described in the table. NSI is unusual in that it synthesizes, but does not secrete, the myeloma K chain. K chain secretion is reactivated in the presence of an H chain provided by a spleen cell[2] (see the table). The types of chains secreted by parental hybridoma lines and their variants may be confirmed by internal radiolabeling and SDS–PAGE and by comparison to the myeloma products. Chains secreted by K, L, or H chain-loss variants of HLK lines are illustrated in Fig. 1.

[3] G. Köhler and M. J. Shulman, *Curr. Top. Microbiol. Immunol.* **81**, 143 (1978).
[4] G. J. Hämmerling, U. Hämmerling, and J. F. Kearney, *in* "Monoclonal Antibodies and T-Cell Hybridomas," pp. 563–571. Elsevier/North-Holland, Amsterdam, 1981.
[5] G. Köhler, H. Hengartner, and C. Milstein, *Protides Biol. Fluids* **25**, 545 (1977).
[6] H. Hengartner, T. Meo, and E. Muller, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4494 (1978).
[7] T. A. Springer, *J. Immunol. Methods* **37**, 139 (1980).

CHAIN-LOSS VARIANTS ARISING FROM HLK NSI × SPLEEN CELL HYBRIDS

Nature of chain loss	Intracellular synthesis	Ig secretion phenotype[a]	K chain assay inhibition	Antigen binding
None	HLK	HLK	+	+
−K	HL	HL	−	+
−L	HK	HK	+	−[b]
−H	LK	L[c]	−	−

[a] NSI does not secrete its K chain in the absence of an H chain.[2]
[b] There may be rare cases in which K chain complements antigen binding activity.
[c] There may be rare cases in which H chain expression is required for L chain secretion; however, this will not affect the radioimmunoassay results.

Previous methods of screening for variants involved the internal radiolabeling and polyacrylamide gel electrophoresis (PAGE) analysis of a large number of subclones.[5,8] However, this is time consuming and expensive. Therefore, a rapid radioimmunoassay specific for idiotypic or V_κ subgroup determinants on the NSI and P3-X63 K chain was developed.[7] The assay measures the ability of hybridoma culture supernatants competitively to inhibit the binding of ^{125}I-labeled anti-P3 V_κ idiotype to HLK or HK IgG coated on plastic Microtiter wells. The target antigen (HLK or HK) and immunogen (P3, which is GK) share only the K chain, rendering the assay specific for this chain. P3 IgG, but not other myeloma proteins, inhibit, demonstrating the idiotype or V_κ subgroup specificity of the absorbed anti-P3 IgG serum (Fig. 2A). HL variants do not inhibit, but HLK monoclonal antibodies in the form of hybridoma culture supernatants give potent inhibition (Fig. 2B). In addition to screening for loss variants, the assay is also useful for typing hybrid lines and for quantitating the amount of K chain secreted (Fig. 2B).

A. P3 K Chain Radioimmunoassay and Loss-Variant Selection[7]

1. Antibodies

Antibodies described here are for use with the NSI and P3-X63 myeloma lines, which were derived from P3 (MOPC 21) and hence have idiotypically identical light chains. Antibodies specific for K or G chains of nonrelated myelomas, e.g., MPC-11, could be obtained by similar procedures. P3 (MOPC 21) IgG may be obtained from serum or ascites of BALB/c mice bearing P3-X63 or MOPC 21 tumors (American Type Cul-

[8] G. Köhler and C. Milstein, *Eur. J. Immunol.* **6**, 511 (1976).

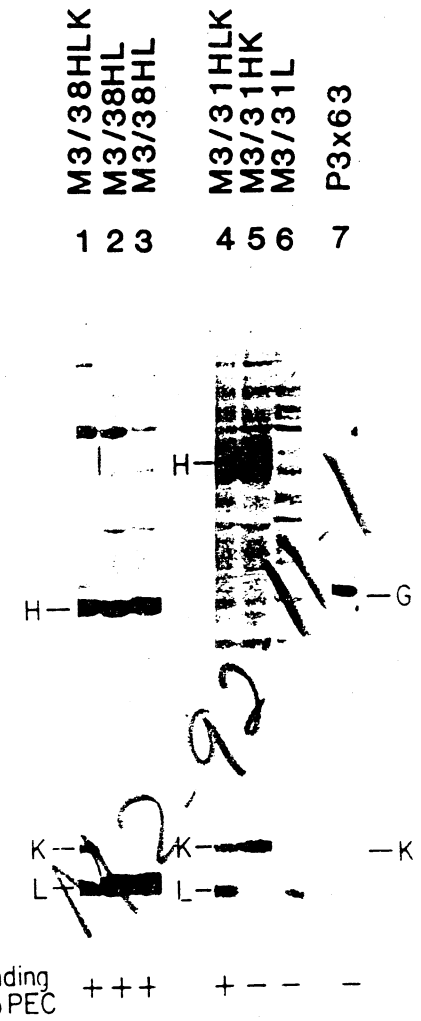

FIG. 1. *SDS–PAGE* of immunoglobulin chains secreted by HLK hybridoma cells and their chain-loss variants. M3/38.1.2 and M3/31.1.1.6 are NSI × rat spleen cell hybrids that secrete HLK antibodies of IgG2a and IgM subclass, respectively, which bind to the Mac-2 antigen[26] on murine peritoneal exudate cells (PEC). Subclones (460 of each line) were tested for K chain loss by the P3–chain radioimmunoassay and for rat L chain loss by radial immunodiffusion against rabbit anti-rat Fab. After labeling with [^{14}C]leucine, secreted products were subjected to SDS–PAGE under reducing conditions and autoradiographed. From M3/38.1.2 (lane 1) were obtained 2 HL variants (lanes 2 and 3), 2 HK variants, and 3 L variants (not shown). From M3/31.1.1.6 (lane 4) were obtained 3 HK variants (one shown in lane 5) and 2 L variants (one shown in lane 6). The L variants are presumably due to H chain loss, which secondarily leads to absence of K chain secretion.[2] The K chain secreted by P3-X63 (lane 7) serves to identify the corresponding chain in the NSI hybrids. NSI was derived from P3 and therefore has an identical light chain. Loss of H or L chain in all cases correlated with loss of antibody activity, measured in a binding assay, whereas loss of K chain did not.

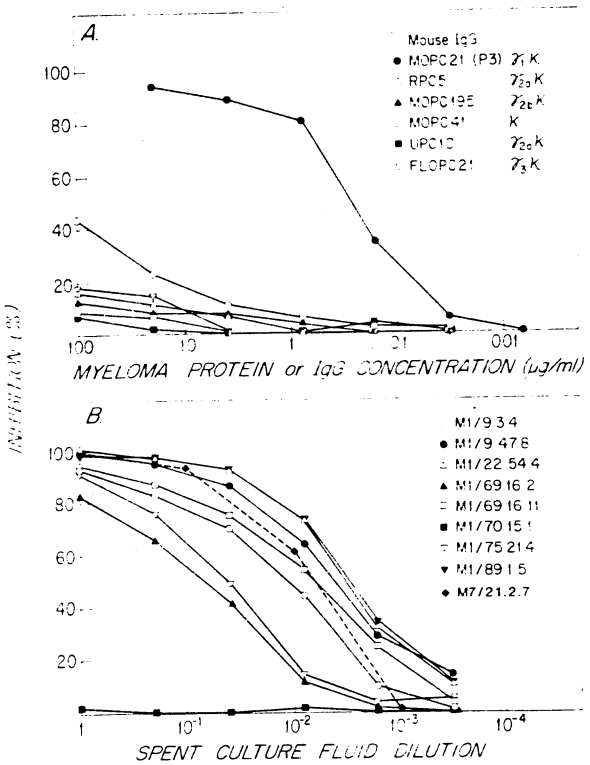

FIG. 2. Characteristics of the P3 K chain assay. (A) Inhibition by MOPC 21 (P3) IgG, other myeloma proteins containing kappa chains, and mouse IgG. (B) Inhibition by products secreted into culture medium by NSI × rat spleen cell hybrids. M1/70.15.1 is an HL line; the others are HLK lines as verified by SDS–PAGE of ^{14}C-labeled secreted products. The solid lines and dashed lines represent the assay carried out with target HLK antigen coated onto sheep red blood cells[7] or Microtiter wells, respectively. The characteristics of the assay are quite similar in both cases.

ture Collection), or from Litton Bionetics. Rabbits are immunized by three monthly injections of 1 mg of P3 IgG in complete Freund's adjuvant distributed to multiple intramuscular sites. Bleeds may be taken biweekly thereafter for several months. The antiserum is absorbed by passing 10 ml through a 4-ml column of rat or mouse serum coupled to Sepharose CL-4B (Pharmacia) (19 mg of protein per milliliter of settled beads). This absorption renders the antiserum specific for P3 IgG idiotypic determinants. Completeness of the absorption should be checked by comparing binding to normal IgG and to K chain-containing MAb in the radioimmunoassay.

2. *Iodination*[9,10]

Materials

Microfuge tube (Sarstedt, No. 701)
Tubing, $\frac{1}{8}$ inch i.d., $\frac{3}{16}$ inch o.d. (Tygon R-3603)
Tubing, 0.05 inch i.d., 0.09 inch o.d. (Tygon S-54-HL)
Tubing connector, $\frac{1}{16}$ inch to $\frac{1}{8}$ inch (Value Plastics, Loveland, Connecticut, No. AC)
Glass wool
Hemostat clamp
Adjustable micropipettor and disposable plastic tips
Sephadex G-25 swollen in phosphate-buffered saline (PBS)
P3 or M1/69 HK IgG, coupled at 2 mg/ml to Sepharose CL-4B
$NaPO_4$, 0.2 *M*, pH 7.5
PBS: 0.8% NaCl, 0.05 *M* $NaPO_4$, pH 7.5
Absorbed anti-P3 IgG serum (anti-P3 idiotype)
Glycine-HCl, 0.1 *M*, pH 2.5
Glycine-HCl, 0.1 *M*, pH 2.5; 1% bovine serum albumin (BSA)
Carrier-free $Na^{125}I$, 1–2 mCi in 1–20 μl
HCl, 0.1 *N*
Chloramine-T, 2 mg/ml, dissolved before use in PBS
Tris-HCl, 1 *M*, pH 8.4

Procedure. Anti-P3 idiotype is purified by adsorption to a P IgG–Sepharose CL-4B or M1/69 IgG–Sepharose CL4B affinity matrix an is iodinated *in situ.*

1. Prepare a minicolumn by cutting off the tip of a microfuge tub pushing a $\frac{1}{2}$ inch long piece of $\frac{1}{8}$ inch i.d. tubing over the bottom, an adding a 6 inch piece of 0.05 inch i.d. tubing with the connector. Th column is mounted in one of the 96 holes formed when the top half of hard-well Microtiter plate is sawed off, and the plate is held in a ringstanc Flow rate is regulated either by lopping the outlet through a hole in th Microtiter plate and adjusting the hydrostatic head, or with a hemostat Tamp a ball of glass wool to the bottom of the column and fill it with PBS Add Sephadex G-25 to make a 50-μl bed, then add a 10-μl bed of P IgG–Sepharose CL-4B.

2. Preelute the column with 1 ml of glycine-HCl, pH 2.5, and was with 2 ml of PBS.

3. Slowly, over 10 min, pass through sufficient anti-P3 idiotype t saturate the column (about 0.2 ml).

[9] L. E. M. Miles and C. N. Hales, *Biochem. J.* **108**, 611 (1968).
[10] L. A. Herzenberg and L. A. Herzenberg, *in* "Handbook of Experimental Immunology (D. M. Weir, ed.), p. 12.1. Blackwell, Oxford, 1978.

4. Wash with 4 ml of PBS, and clamp off the effluent when no PBS remains above the Sepharose.

5. The iodination is carried out in a properly equipped fume hood. Prepare ^{125}I by adding 5 μl of 0.2 *M* $NaPO_4$, pH 7.5, then sufficient 0.1 *N* HCl to neutralize the NaOH in the ^{125}I.

6. Add 5 μl of 2 mg/ml chloramine-T to the ^{125}I, mix, immediately transfer with the micropipettor to the Sepharose bed in the column, and suspend the Sepharose layer by gentle stirring with the plastic tip for 1 min.

7. Wash with 2 ml of PBS (collect as flow-through)

8. Elute with 2 ml of 0.1 *M* glycine-HCl ph 2.5, + 1% BSA into a tube containing 1 ml of 1 *M* Tris-HCl, pH 8.4, and mix by vortexing (collect as eluate).

9. Determine with a portable radioactivity monitor the relative proportion of counts in the flow-through, the eluate, and those remaining on the column., About 30% incorporation into the eluate is normally achieved.

10. Dialyze the eluate overnight versus 1 liter of PBS, dilute to 2000–20,000 cpm/μl with 5% BSA in PBS, and store at −80° in 2-ml aliquots.

3. Subcloning

Hybridoma lines are allowed to grow for 2–4 months after the previous subcloning to allow variants to arise. Soft agar (0.3%) cloning of 1000 and 3000 cells/100-mm petri dish and growth of hybridoma cells are carried out as described elsewhere.[1] After 7–10 days, agar plugs containing single clones are transferred to 96-well microculture plates (Costar, Cambridge, Massachusetts) containing 0.2 ml of medium per well. A blank agar plug is transferred as a control. Since K chain loss variants are found at a frequency of about 10^{-2}, about 400 clones are picked. Clones grow at different rates. After some clones grow sufficiently to lower the pH of the medium (phenol red turning orange), about two-thirds of the medium in every well is aspirated and replaced with fresh medium every 2 days. At least three changes are made before assay, to allow most of the residual K chain from the agar plug to be diluted out. After this number of changes, HL variants and the control agar plug may inhibit the P3 K chain assay slightly, but will be clearly distinguishable from HLK lines. For assay, about 100 μl of culture fluid are transferred into sterile V-well Microtiter "master" plates (Flow Laboratories, No. 76-222-05) with a 12-channel, 50–200 μl adjustable micropipettor (Flow Laboratories, No. 77-889-00) equipped with autoclaved plastic tips. One set of 12 tips is used for an entire Microtiter plate, rinsing twice with sterile distilled water in a

reservoir (Flow Laboratories, No. 77-824-00) and touching off residual solution onto an autoclaved paper towel between each transfer.

4. Radioimmunoassay[11,12]

Materials

^{125}I-anti-P3 idiotype
Purified HK or HLK MAb, 10 μg/ml in PBS (M{1/{69 HK IgG may be obtained from Boehringer-Manheim, Indianapolis, Indiana, or the M{1/{69 HK cell line from the American Type Culture Collection, Rockville, Maryland).
Normal IgG, 10 μg/ml in PBS
Fetal calf serum (FCS), 10% in PBS
BSA, 1% in PBS
Micropipettor, 50–200 μl, 12 channel (Flow Laboratories, No. 76-222-05)
Soft-well Microtiter radioimmunoassay (RIA) plates (Costar No. 2595)
Syringe, 250 μl, with repeating dispenser for 5-μl aliquots (Hamilton, Reno, Nevada)
Hot wire Microtiter plate cutter (D Lee, 932 Kintyre Way, Sunnyvale, California)
Adhesive tape (plate sealing tape, Cooke Laboratories, Alexandria, Virginia)
Manifold, 12 needle (Cooke No. 300-5) and Cornwall syringe for delivering wash buffer, a similar 12-needle manifold for aspiration, and a Microtiter plate shaker (Cooke No. 2-225-06) are handy but optional pieces of equipment.

Procedure

1. The HLK or HK target antigen in PBS is transferred with the 12-tip pipettor to soft-well Microtiter plates (50 μl/well) and allowed to adsorb to the wells for 2 hr at room temperature or overnight at 4°. The use of an HLK or HK target antigen, rather than P3 IgG or HGLK, ensures that the subset of anti-idiotype antibodies specific for the P3 K chain, but not the G chain, are included in the assay. Wells coated with normal IgG and with 1% BSA are included as controls.

2. Wells are emptied by aspiration, and residual binding sites on the plastic are saturated with 120 μl of 1% BSA in PBS for 5 min; the wells are then washed twice more with the same solution.

[11] T. A. Springer, A. Bhattacharya, J. T. Cardoza, and F. Sanchez-Madrid, *Hybridoma*, in press (1982).
[12] G. H. Parsons, Jr., this series, Vol. 77, p. 224.

3. Aliquots to be tested (50 μl) are transferred with the multichannel pipettor from the "master" Microtiter plate to the soft-well assay plate. Serial dilutions in 10% FCS–PBS of supernatant from the parent hybridoma are also tested to establish a standard curve. The diluent for the competition step contains 10% serum, which gives a lower background than 1% BSA.

4. Aliquots of 5 μl of ^{125}I anti-P3 idiotype are added with the Hamilton syringe (for highest accuracy the aliquot is added while the plate is shaking). The plate is shaken and allowed to stand $\frac{1}{2}$ hr at room temperature.

5. The plates are aspirated and washed 3 times with 1% BSA–PBS. Residual liquid is removed from plates by throwing them upside down onto absorbent paper.

6. Adhesive tape is applied to the bottom of the soft-well plate, and the top is cut off by pushing the plate horizontally through the hot wire cutter, leaving the isolated wells adhering to the tape. Wells are plucked off and gamma-counted.

7. Percentage of inhibition is calculated as

$$(1 - e/t)/(1 - c/t) \times 100$$

where e = experimental cpm bound in the presence of inhibitor to antigen-coated wells, c = control cpm bound in the presence of 10% FCS–PBS to BSA-coated wells, and t = total cpm bound in the presence of 10% FCS–PBS to antigen-coated wells. Total cpm bound should be 10–30% of input cpm.

5. Further Characterization of Variant Subclones

If HK variants are also desired, parallel testing of the subclones for loss of antigen-binding can be carried out.

In testing for HL variants with the P3 K chain assay, it is common to find a large proportion of subclones that give complete inhibition (HLKs) and about 1% that give moderate to weak inhibition (HLs). The latter are transferred to 2 ml of medium in 24-well plates. True HL variants are found to give no inhibition after further growth and media changes.

Both H and K chain loss can lead to absence of K chain secretion (see the table). Therefore it is important to confirm the nature of the chain loss both by testing for antibody activity and by internal radiolabeling and PAGE of the secreted chains (Fig. 1). Specific light chains (of mouse or rat) can almost always be resolved from the myeloma K chain by discontinuous SDS–PAGE.[13] Separation may be due to charge differences, which are important in the stacking gel, as well as to size differences between different V_L regions.

[13] T. Springer, G. Galfrè, D. S. Secher, and C. Milstein, *Eur. J. Immunol.* **8**, 539 (1978).

Subclones showing the desired chain loss are expanded by further growth for collection of culture supernatants and liquid N_2 storage of frozen cells.[1]

II. Quantitation of Monoclonal Immunoglobulins

Immunoassays are useful for determining the concentration of monoclonal antibodies secreted into culture medium, ascites, or serum and to guide purification. The accurate determination of monoclonal Ig concentrations by immunoassay presents special problems. The use of anti-IgG sera is inappropriate, since such sera contain a high proportion of antibodies directed to subclass-specific determinants on the Fc region. In inhibition assays, the proportion of inhibitable anti-IgG antibodies thus depends on the subclass of the MAb being tested (Fig. 3B). This can lead to artifactually low estimates of monoclonal immunoglobulin concentrations. Anti-Fab sera show much less subclass preference. Rabbit anti-Fab sera is routinely used in this laboratory in single radial immunodiffusion (Mancini assay[14]) for the determination of mouse and rat MAb concentrations. This assay takes very little time to carry out and has the convenience that the Mancini plates may be stored for at least up to a year before being used.

Antisera to Fab fragments prepared from whole rat or mouse IgG are primarily specific for kappa chain determinants. It should be cautioned that these sera almost invariably do not react by radial immunodiffusion with lambda chain-containing monoclonal immunoglobulins. About 10% of mouse and rat immunoglobulins contain lambda chains, and we have found about the same proportion in rat anti-mouse cell surface MAb.[11] Antisera to lambda chains or the appropriate heavy-chain subclass must be used to quantitate these MAb.

In working with antisera to rat Fab or rat kappa chains, it is important to take the kappa allotype into account. Two allelic forms of rat kappa chains have been defined, RI-1a in DA, ACI, and Fisher rats, and RI-1b in Lewis, BN, LOU, and Wistar rats.[15–17] The two allotypes differ by 11 amino acids in the kappa chain constant region. This is a very large difference and is comparable to the interspecies difference between mouse and rat RI-1a kappa chains of 14 residues.[18,19] Therefore, the rat

[14] G. Mancini, A. O. Carbonara, and J. F. Heremans, *Immunochemistry* **2**, 235 (1965).
[15] G. A. Gutman and I. L. Weissman, *J. Immunol.* **107**, 1391 (1971).
[16] A. Beckers, P. Querinjean, and H. Bazin, *Immunochemistry* **11**, 605 (1974).
[17] G. A. Gutman, *Immunogenetics* **5**, 597 (1977).
[18] G. A. Gutman, *Transplant. Proc.* **13**, 1483 (1981).
[19] H. W. Sheppard and G. A. Gutman, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7064 (1981).

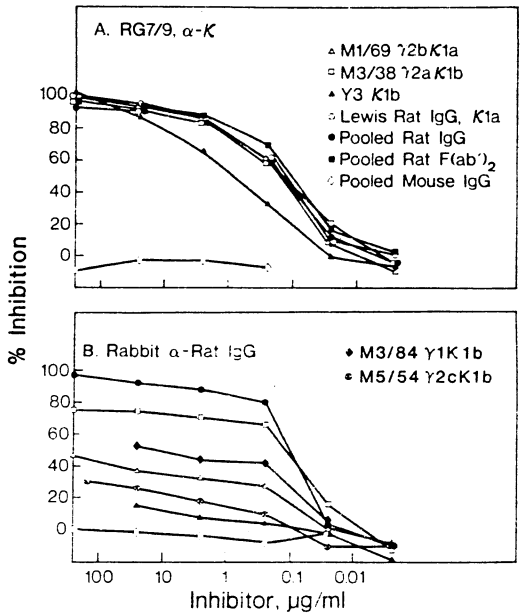

FIG. 3. Radioimmunoassay determination of rat monoclonal immunoglobulin concentrations.[11] Serial dilutions of rat monoclonal immunoglobulins were incubated with ^{125}I-labeled, monoclonal mouse anti-rat kappa chain (panel A), or affinity-purified rabbit anti-rat IgG (panel B). The residual binding capacity of the ^{125}I-labeled antibodies was then measured after transfer to rat IgG-coated soft-well plates. (A) Kappa 1a and 1b allotype-bearing monoclonal immunoglobulins and whole IgG all inhibited the monoclonal anti-rat K chain antibody identically, showing that it can be used to determine monoclonal antibody concentration. The monoclonal anti-kappa antibody was prepared against whole IgG and apparently has a lower affinity for free kappa chains. (B) Inhibition by the monoclonal immunoglobulins of ^{125}I-labeled rat IgG plateaued at submaximal levels, showing that each can inhibit only a subpopulation of the anti-rat IgG antibodies with the appropriate specificity.

IgG preparation used to construct the standard curve for radial immunodiffusion or radioimmune assays, and the samples being analyzed, should always be of the same allotype. In contrast to the rat, mouse kappa chains do not express allotype differences.

Radioimmunoassay is an alternative to single radial diffusion for determining monoclonal immunoglobulin concentrations. This laboratory has prepared a monoclonal antibody that has equal affinity for rat kappa chains of 1a and 1b allotypes.[11] It is an excellent reagent for determination of monoclonal immunoglobulin concentrations (Fig. 3A) and for use in the indirect binding assay and many other applications.[11] Monoclonal antibodies with specificity for rat IgG subclasses and kappa allotypes,[11] and with specificity for mouse kappa chains and IgG subclasses are also avail-

able.[20] Since many of the monoclonal anti-IgG reagents that have subclass specificity are directed to Fc region determinants,[11] they are also useful for monitoring or effecting the removal of Fc fragments and undigested IgG from Fab or $F(ab')_2$ preparations.

A. Single Radial Immunodiffusion Determination of Monoclonal Immunoglobulin Concentrations

1. Anti-Rat (or Mouse) Fab Serum

To purified rat (or mouse) IgG, 10–20 mg/ml in PBS, 2 m*M* EDTA, is added 1/100 by weight of papain (Worthington, Freehold, New Jersey) and 2-mercaptoethanol to bring to 10 m*M*. Digestion is carried out under N_2 for 18 hr at 37°, and sodium iodoacetate and Tris-HCl, pH 8.6, are added to 11 m*M* and 20 m*M*, respectively, to stop digestion. Fab and Fc fragments are separated from small peptides and any residual undigested IgG by Sephadex G-200 filtration. The Fab and Fc pool is dialyzed versus 0.05 *M* Tris-HCl, pH 8.0, and applied at 6 mg of protein per milliliter of bed volume to a DEAE-cellulose column (DE-52, Whatman) equilibrated with the same buffer. The column is washed with 0.05 *M* Tris-HCl, pH 8.0, and eluted with a gradient of 0 to 0.2 *M* NaCl in the same buffer. Separation of Fab from Fc is monitored by immunoelectrophoresis[21,22] or radioimmunoassay. Pure Fab should elute in the flow-through.

Antisera to Fab fragments are prepared as described in Section I,A,1.

2. Single Radial Immunodiffusion[14,22,23]

In single radial immunodiffusion, antigens in a central well diffuse into antiserum-containing agar. When equilibrium is reached, the area bounded by a precipitate ring around the well is proportional to the amount of antigen applied.

To determine the proper amount of antiserum to use, the procedure described below is scaled down for use with nonfrosted microscope slides. Each slide receives agar containing a different amount of antiserum, three 3 mm in diameter wells are punched, and 8-μl aliquots of IgG at 200, 100, and 50 μg/ml are applied. The lowest antiserum concentration

[20] D. E. Yelton, C. Desaymard, and M. D. Scharff, *Hybridoma* **1**, 5 (1981).
[21] D. R. Stanworth and M. W. Turner, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), p. 6.25. Blackwell, Oxford, 1978.
[22] O. Ouchterlony and L.-A. Nilsson, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), p. 19.10. Blackwell, Oxford, 1978.
[23] J.-P. Vaerman, this series, Vol. 73, p. 291.

that still gives well-defined rings is selected for use. In our experience, this concentration will give rings of about 8.5 mm in diameter with 100 μg of IgG per milliliter.

To prepare the plates, an appropriate volume of antiserum (about 100 μl if high-titered) is mixed with 20 ml of 1% agar (Difco) in 0.01 *M* Tris-HCl pH 7.8, 0.14 *M* NaCl, 0.1% NaN_3 at 55°; 19 ml are spread evenly over a prewarmed, level, 4 × 5 inch glass plate and allowed to solidify. Plates may be stored at least 1 year in sealed containers (Tupperware) containing paper towels wetted with the same buffer. A 5 × 7 matrix of holes 3 mm in diameter is punched in the agar with a No. 1 cork borer attached to an aspirator, and 8-μl samples are applied, avoiding overflowing. Standards of mouse IgG or of the appropriate kappa chain allotype of rat IgG at 200, 100, 50, 20, and 10 μg/ml are applied to each plate. Unknowns are applied at several different dilutions guessed to give concentrations of about 100 μg/ml. High concentrations can cause artifacts in neighboring wells.[23] Diffusion is allowed to occur for 2 days in a humid atmosphere at room temperature. Ring diameter is measured using a viewing stand. The stand is a 9 inch × 7½ inch piece of ¼ inch plywood with a 5½ inch hold cut out of its center, supported on 9 inch long dowel legs. The stand is placed in a darkroom on a light box with a piece of black paper directly beneath the stand to provide indirect illumination. Agar is cut from the corners of the plate that touch the stand, and the plate is inverted and placed on the stand. The agar remains adherent to the plate. Ring diameter is measured in two perpendicular directions through a measuring magnifier (Bausch and Lomb No. 81-34-38) placed on the back of the plate. A calibrating viewer, Model 2743, is also available from Transidyne General Co., Ann Arbor, Michigan. The standard curve is constructed by plotting the product of the two ring diameters against antigen concentration.

B. Radioimmunoassay for Quantitating Monoclonal Immunoglobulins[11]

Monoclonal mouse antibodies to rat kappa chains (RG7/9) and rat IgG subclasses 1 (RG11/39), 2a (RG7/1), and 2b (RG7/11)[11] can be obtained from Boehringer-Mannheim (Indianapolis, Indiana) or by growth of the cell lines (American Type Culture Collection, Rockville, Maryland). Monoclonal rat antibodies to mouse kappa chains and IgG subclasses[20] are available from New England Nuclear (Boston, Massachusetts). Antisera to Fab fragments are prepared as described in Sections II,A,1 and I,A,1. Antibodies can be affinity purified and radioiodinated on Sepharose CL-4B conjugated to the appropriate antigen, as described in Section I,A,2. For mouse monoclonal antibodies, we have found it to be more convenient to purify milligram quantities by *Staphylococcus aureus* pro-

tein A affinity chromatography[24] and iodinate 10-μg quantities using chloroglycoluril.[25]

Monoclonal immunoglobulins in culture supernatants, ascites, or other form and normal IgG standards are serially diluted in 10% FCS–PBS in hard-well Microtiter plates. IgG, 10 μg/ml in PBS, is adsorbed to soft-well Microtiter plates. The competition assay is then carried out as described in Section I,A,4. Alternatively, an equilibrium inhibition assay may be carried out. The ^{125}I-MAb (5 μl) is mixed with antigen dilutions (50 μl) in hard-well plates, the plates are sealed with tape and allowed to stand overnight at 4°, and then 40-μl aliquots are transferred to the antigen-coated soft-well plates, and the assay is continued as described in Section I,A,4.

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[12] Indirect ^{125}I-Labeled Protein A Assay for Monoclonal Antibodies to Cell-Surface Antigens

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The hybridoma technique of Köhler and Milstein[1,2] has greatly facilitated the identification and characterization of cell-surface antigens, for which monoclonal antibodies have many advantages over conventional serological reagents. However, some of the methods commonly used to test antisera are unsuitable for use with monoclonal antibodies, either because they fail to detect antibodies of certain immunoglobulin isotypes or because they are insensitive to antibodies bound to a single antigenic determinant. Mouse IgG1 antibodies, for example, are not detected by complement-dependent cytotoxicity,[3] and IgG2a antibodies to a single

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