142-01

18

Solid-Phase Radioimmune Assays

Theta T. Tsu and Leonore A. Herzenberg

18.1 INTRODUCTION

Radioimmune assays (RIAs) combine the exquisite specificity of antigen-antibody reactions with the quantitative accuracy of isotope determinations. They allow sensitive measurement of antibody or antigen concentrations, even in unpurified preparations. Many procedural variations in the RIA have been developed to meet particular clinical or research needs, but the underlying principle of all assays is the same: Bound radioactivity is used as an index of the amount of antigen bound to a given quantity of antibody or of the amount of antibody bound to a given quantity of antigen.

Radiolabel may be bound either directly in the antigen-antibody complex by radiolabeling one of the participants of the reaction (antibody or antigen) or it may be bound indirectly by radiolabeling a reagent, generally a second antibody, which reacts with one of the unlabeled participants to provide an index of the amount of the participant bound in the initial antigen-antibody complex. In each case, the radiolabel bound to the complexes must then be separated from unbound radiolabeled material so that the amount of bound radioactivity can be determined.

In this chapter, we describe several variations of the "solid-phase" RIA in which one of the reactants is immobilized on the plastic inner surface of a 96-well (Microtiter) polyvinylchloride plate to facilitate separation of the antigen-antibody complexes from unbound material. The use of this solid-phase technology has greatly improved the sensitivity, specificity, and practicality of the RIA and has thus substantially expanded the attractiveness of this type of assay for many purposes. The examples we have chosen are intended to demonstrate the versatility of the solid-phase RIA and to serve as prototypes for assays that can be adapted to meet specific needs.

The ultimate precision and sensitivity of the RIA depend on the selection and preparation of suitably specific antibody reagents and the careful determination of optimal reagent concentrations. While guidelines for the preparation and use of reagents are presented in this chapter, it is necessary to determine empirically the exact experimental conditions for each antibody-antigen system. Generally accurate measurements for antigen or antibody in the nanogram range can be obtained with careful technique. Similar results with perhaps slightly less sensitivity and precision may be obtained in assaying the reactions of cell surface determinants and their specific antibodies when employing cell-binding RIA methods (analogous to solid-phase RIAs).

The cell-binding assays require centrifugation to separate the complexes of cells and bound reagents from unbound material. Aside from this procedural modification, however, cell-binding RIAs and solid-phase RIAs follow essentially the same general protocol. In the introductory text that follows, we present a general description of the procedure to provide an overview of the assay. In subsequent sections, we describe in detail specific protocols for preparing reagents and for using several variations of these methods.

1. Coating the Plate

Proteins and other macromolecules in solution tend to stick nonspecifically to plastic. The adsorption is essentially irreversible in buffered salt solutions such as phosphate-buffered saline (PBS), pH 7.4. Thus, incubation of an antigen or antibody solution in Microtiter wells results in deposition of a coat which persists throughout repeated washing.

The amount of antigen or antibody deposited depends on its concentration in solution, its inherent "stickiness", and the amount of contaminating material in solution capable of competing for sites on the plastic. Purified coating reagents yield more efficient coating. Titration curves based on the binding of the radiolabeled reagent to be used in the assay should be obtained to determine the most efficient coat concentration, taking into consideration the potential problem of increases in assay backgrounds due to nonspecific binding of radiolabeled reagents, which may occur at high coat concentrations.

2. Blocking the Plate

After the coat has been deposited, the plate must be "blocked" to prevent nonspecific adsorption of subsequently added reagents. Several washings of the coated wells with a concentrated protein solution, for example, 1% bovine serum albumin in PBS (RIA buffer) are quite satisfactory for this purpose. No incubation time is required for this step other than that involved in the washing process.

3. Washing the Plate

Plates are washed by filling the wells and dumping or "flicking" the contents of the wells into a sink or other suitable receptacle. The last drops of wash fluid are removed by throwing the plate, face down, onto several layers of absorbent material on a table.

4. Binding the Radiolabeled Reagent

Here, assays adapted for different purposes vary considerably (see Table 18.1):

TABLE 18.1
Adaption of solid-phase radioimmune assays for different purposes

Well coat	First step	Second step	Use	Refer to
			One-Step Assay	
Ag	^{125}I-Ab	—	Measure reactivity of antibody	Section 18.3
Ab	^{125}I-Ag	—	or antigen	
Ag	Ag + ^{125}I-Ab	—	Measure or compare Ag-blocking assay	Section 18.5
Ab	Ag + ^{125}I-Ag	—	Measure or compare Ab-blocking assay	Section 18.5
			Two-Step Assay	
Ag	Ab	^{125}I-Ig	Measure reactivity of Ab or Ag; determine isotype or allotype of Ab	Section 18.4
Ab	Ag	^{125}I-Ab (to Ag; must not react with Ab)	Measure reactivity of Ab or Ag	
Ag	Ab + Ag	^{125}I-anti-Ig (must not react with Ag)	Measure or compare Ag-blocking assay	

a. In direct binding (one-step) assays, labeled reagent is simply added to the coated well and allowed to incubate.

b. In indirect (two-step) assays, unlabeled "first-step" antibody (or antigen) is incubated in the well for a period of time, and the well is then washed with RIA buffer to remove all unbound material. Radiolabeled reagent specific for the first-step reagent is then added and incubated.

c. In blocking assays, a solution containing material potentially similar to the coating antigen (or antibody) is mixed with the radiolabeled reagent and the mixture is added to the well and incubated. Alternatively, the blocking agent and then the radiolabeled reagents are added sequentially to the well prior to incubation.

In each procedure, the final incubation allows binding of radiolabeled reagent either to the coat antigen or to the first-step reagent bound to the coat antigen. Excess radiolabeled reagent is removed from the wells after incubation and the wells are washed with RIA buffer to remove any additional unbound label.

5. Counting the Radioactivity Bound to the Wells

In general, ^{125}I-labeled reagents are used for these assays. The wells need only be severed from each other and dropped into carrier tubes, which can be counted in a gamma scintillation counter.

Severing the wells of the soft plastic plates recommended for use here can be accomplished simply by cutting them apart with a scissors or knife; however, if large numbers of wells are to be processed, wells can be severed by a more orderly and less time-consuming procedure. Adhesive plastic sheets (designed originally as nonpermeable covers for the Microtiter plates) can be firmly adhered to the *bottom* of the plates such that each well is anchored at its base. The top of the plate can then be cut away with a hot wire, leaving the anchored wells free to be plucked from their adhesive support and placed in counting tubes. A hot wire cutter has been especially designed for this purpose (see Materials and Reagents, Section 18.3).

6. Converting Counts to Quantitative Data Comparable from Experiment to Experiment

Since ^{125}I decays with a half-life of 60 days, expressing data as counts per minute (cpm) is often inadequate for a series of experiments conducted over a long period of time. Similarly, because assay conditions may vary slightly from day to day, raw count values may be misleading. We have found it helpful to include a standard curve with each assay and to express data in terms of standard equivalents (mg or μl) per milliliter of test serum. Use of standard curves also allows correction for nonlinearity of binding and thus gives a more accurate representation of the amount of antibody (or antigen) in the test serum.

18.2 PREPARATION OF RADIOLABELED REAGENTS FOR RIA

Editors' Note: Safety precautions necessary for work with sodium ^{125}iodide and iodinated material are described in Section 14.2.

Any of the standard iodination procedures are satisfactory for preparing ^{125}I-RIA reagents as long as most of the labeled protein in the preparation proves to be active in the specific binding reaction. We use a solid-phase radiolabeling method in which the antibody is iodinated while bound to antigen insolubilized by covalent binding to Sepharose beads, as described by Miles and Hales (1968). This method allows minimal losses in the labeling of small amounts of antibody because the antibody binding sites are protected during the labeling, and absorptions and elutions can be carried out in the presence of protein (e.g., BSA) to guard against nonspecific losses on glassware during the procedure. In addition, the method is reliable, reproducible, and economical since it does not require a separate antibody purification step prior to labeling.

Reagents prepared in this fashion usually contain more than 60% ^{125}I-labeled specifically bindable antibody; however, occasionally the procedure yields substantially less bindable antibody. In these instances, the labeled antibody is readsorbed to the antigen-Sepharose and re-eluted to bring the reagent up to standard for use in the RIA. on

Soluble iodination is also used in our laboratory, mainly when antigen-Sepharose conjugates are unavailable (e.g., for labeling antibody reactive with cell surface determinants or when determination of the specific activity of the labeled protein is required).

The procedure described below is for labeling antibodies to immunoglobulins. Appropriate modifications are required if other kinds of antibodies or if antigens are to be labeled.

MATERIALS AND REAGENTS

- Antiserum to be labeled: Antiallotype and other antisera are made as described by Herzenberg and Herzenberg (1978a).
- Antigen conjugated to Sepharose 4B: Couple purified antigen (myeloma protein) to Sepharose using CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) and the method described in a booklet available from Pharmacia—*Affinity Chromatography: Principles and Methods*. The protein-to-Sepharose ratio is 5 mg/ml gel. Store conjugate in PBS with 0.02% NaN_3 at 4°C.
- Phosphate-buffered saline (PBS; see Appendix A.8)
- Airfuge, air-driven ultracentrifuge (Beckman Instruments)
- Sero-Fuge (Clay Adams, #0511)
- PD-10 column, Sephadex G-25M (Pharmacia Fine Chemicals)
- Pipetman, 20 μl and 200 μl (Rainin Instrument Co.)
- Tube, 10 × 75 mm
- Pasteur pipette, sealed, long tip (used as a stirring rod)
- Gamma counter
- Buffers and solutions
  - 0.5 M phosphate buffer, pH 7.5: Dissolve 1.12 g KH_2PO_4 and 14.96 g Na_2HPO_4, in 100 ml distilled water. For 0.05 M phosphate buffer, dilute 0.5 M buffer 1 : 10 with distilled water. change
  - 0.2 M glycine-HCl buffer, pH 2.3: Add 1.5 g glycine to approximately 90 ml distilled water, and adjust to pH 2.3 with concentrated HCl; add distilled water to bring volume up to 100 ml) ")" to a period.
  - Bovine serum albumin (BSA): 1% (w/v) in PBS, containing 0.02% (w/v) NaN_3. Caution: Sodium azide is extremely toxic.
  - Trichloroacetic acid (TCA): 15% (w/v)
  - Chloramine "T": 10 mg/5 ml of 0.05 M phosphate buffer, pH 7.5
  - Sodium metabisulfate: 10 mg/5 ml of 0.05 M phosphate buffer, pH 7.5 "i" metabisulfite
  - Sodium ^{125}I in 0.1 M NaOH, carrier-free: 10 mCi/0.1 ml

PROCEDURE

1. Deaggregate antiserum in Airfuge (22 psi for 10 min).
2. Place 20 μl immunoadsorbent in a 10 × 75-mm tube. Wash once (tube volume) with 0.2 M glycine-HCl, pH 2.3, by centrifugation in Sero-Fuge for 1 min.
3. Wash immunoadsorbent 2 times with PBS to neutralize (pH should be approximately 7.2). Pellet immunoadsorbent by centrifugation.
4. Add 75 μl of antiserum to the pelleted immunoadsorbent. Incubate at room temperature for 20 minutes (mix twice over the 20-minute period).
5. Wash Sepharose 3 times with PBS. Pellet Sepharose by centrifugation.
6. Add 50 μl of 0.5 M phosphate buffer, pH 7.5, followed by 1 mCi ^{125}I (use within 2 weeks of arrival). Then add 10 μl chloramine "T". Mix for 30 seconds at room temperature with a sealed, long-tip Pasteur pipette.
7. Add 10 μl sodium metabisulfite solution. Mix for 30 seconds.
8. Add 1.4 ml 0.2 M glycine-HCl, pH 2.3, and pipet mixture onto a PD-10 column (equilibrated with 25 ml of 1% BSA in PBS and 0.02% sodium azide). Discard effluent.
9. Rinse tube with 1.0 ml of 0.2 M glycine-HCl, pH 2.3, and wash onto PD-10 column. Discard effluent (void volume is approximately 2.5 ml).
10. Add 3.0 ml of PBS onto the PD-10 column. Collect the effluent (which contains the radiolabeled antibody).
11. Determine the percent of radiolabel precipitable by TCA: percentage
    a. Place a 2-μl sample of the radiolabeled antibody into 150 μl 1% BSA in PBS. Count sample in gamma counter (Count A).
    b. Add 150 μl 15% TCA into counted sample (a) to precipitate protein. Centrifuge sample (290 × g, 10 min) and remove 75 μl of the supernatant. Count sample in gamma counter (Count B). should be supernate

adjective form

$$\% \text{ TCA ppt} = 100\% - \frac{(4 \times B \times 100\%)}{A}$$

12. Store radiolabeled antibody at 4°C.

COMMENTS

1. When recovered sample is reapplied to a second Sepharose immunoadsorbent and eluted, 30–50% is usually recovered.
2. ^{125}I-labeling in solution: Labeling with ^{125}I in solution is identical to solid-phase ^{125}I-labeling except that steps 2–5 (immunoadsorbant binding) are skipped. Also, PBS can be substituted for the 0.2 M glycine-HCl, pH 2.3, eluting buffer in steps 8 and 9. This method can be used when there are sufficient quantities of purified proteins. We routinely use this method to label: (a) purified mouse myeloma protein; use approximately 7.5 μl at 20 mg/ml = 150 μg; (b) staphylococcal protein A; use 10 μl at 1 mg/ml = 10 μg.

18.3 ONE-STEP SOLID-PHASE RIA: SPECIFICITY TESTING OF ^{125}I-LABELED ANTIALLOTYPE REAGENTS

After labeling an antiallotype sera, we test the specificity of the labeled antiallotype by coating 96-well Microtiter plates with myeloma proteins of known class and allotype (e.g., Ig-1a, Ig-1b, Ig-4a, Ig-4b) and measuring the binding of the ^{125}I-labeled antiallotype. When properly prepared, the reagent should bind only to the appropriate myeloma protein.

If the reagent appears to be nonspecific (i.e., binds to the wrong myeloma protein), the coat concentration of the myeloma protein may be at fault. Purified myeloma proteins are frequently contaminated with small amounts of other Ig; thus, too high a coating concentration may result in coating the wells with a sufficient amount of a contaminating Ig class to allow detection of antibody bound to the contaminant. For example, wells coated with a 1 mg per milliliter solution of Ig-1a myeloma protein contaminated with 1% Ig-1b (e.g., from growth of the tumor in BALB/c Ig^b mice) will bind significant levels of ^{125}I-anti-Ig-1b because of the contaminant. (This antibody is known not to bind to Ig-1a because of the mouse strain combination used in the immunization.) Reduction of the coating concentration to 0.01 mg per ml in this case prevents detection of binding to the contaminant but does not interfere with detection of binding to the Ig-1a myeloma protein by reagents specific for it (e.g., anti-Ig-1a).

MATERIALS AND REAGENTS

Myeloma proteins: Ascites and sera from myeloma-bearing mice are purified by ammonium sulfate precipitation (Section 12.2) and column chromatography (e.g., DEAE Sephadex CH-6B or protein A-Sepharose CL-4B; Pharmacia Fine Chemicals; see Sections 12.3 and 17.9).

^{125}I-labeled antiallotype sera

Phosphate-buffered saline (PBS), pH 7.4 (Appendix A.8)

Microtiter plates, "U" bottom, disposable, flexible, nonsterile (Cooke Laboratory Products, 96-2311 plates, #220-24)

Parafilm

Pipetman, 20 μl and 200 μl (Rainin Instrument Co.)

CHUX disposable diapers, used as absorbent paper for drying plates

Pasteur pipettes or apparatus for vacuum aspiration

Hot wire cutter (D. Lee, Inc.)

Gamma counter

RIA buffer, 1% bovine serum albumin (BSA) in PBS, 0.02% NaN_3: For 20 liters, add 200 g BSA (Fr V) to 20 liters PBS. Stir slowly until BSA completely dissolves. Filter solution by positive pressure through a 0.45-μm Millipore filter. Collect into 500-ml bottles (500 ml/bottle), adjust to pH 7.4, and add 1.0 ml of 10% NaN_3 to each bottle. Store at 4°C. Caution: Sodium azide is extremely toxic.

PROCEDURE

1. Dilute myeloma proteins (e.g., Ig-1a, Ig-1b, Ig-4a, Ig-4b) in PBS to subsaturating coating concentrations (0.1 mg/ml–0.01 mg/ml).
2. Add 50 μl of protein solution to an appropriate number of wells. All tests should be done in duplicate. Put RIA buffer in 2–4 wells for negative controls.
3. Cover the plate with Parafilm and incubate for 1 hour at room temperature.
4. Dump fluid from the wells and wash wells 3 times with RIA buffer.
5. After dumping the third wash, dry the plate by throwing it face down onto absorbent paper.
6. Add 20 μl of ^{125}I-labeled antiallotype reagent.
7. Cover plate with Parafilm and incubate 1 hour at room temperature.
8. Remove radioactive supernatant from the plate by vacuum aspiration (or with a Pasteur pipette) and discard the supernatant appropriately. Wash plate 3 times with RIA buffer and dry as above.
9. Cut wells apart with hot wire cutter or scissors and count in gamma counter (as described in the introduction to this chapter).

18.4 TWO-STEP SOLID-PHASE RIA: ESTIMATION OF ISOTYPE AND ALLOTYPE REPRESENTATION IN AN ANTIBODY RESPONSE, E.G., RESPONSE TO THE DINITROPHENYL HAPTEN (DNP) ON A CARRIER PROTEIN

Two-step solid-phase RIAs are easily adaptable to the measurement of antibody responses to soluble antigens and cell surface determinants. Since these assays are very sensitive, very small amounts of sera from responding animals (or of supernatant from hybridoma cultures; see Chapter 17) are required for each test; however, because of this sensitivity, care must be exercised to assure that samples are adequately diluted (in RIA buffer) to place them within the accurate range of the assay (i.e., well below saturation). Usually, this range falls within the reasonably linear portion of the standard curve (titration of purified antibody or standard antiserum) that is run along with each assay to provide a reference for expressing antibody levels in the test sera.

For these two-step solid-phase assays, antigen is coated on (or placed in) the wells of 96-well Microtiter plates. Coating concentrations below 100 μg/ml are usually used to decrease assay backgrounds. After the appropriate washing of coated wells, dilutions of test or standard first-step antisera are incubated in the wells. After appropriate washing, ^{125}I-labeled anti-Ig (second-step) reagents are incubated in the wells to provide an index of the amount of first-step antibody bound. Choice of the second-step reagent(s) determines which isotype(s) or allotype(s) represented in the response are measured.

We have used this type of RIA extensively to measure the adoptive secondary response of allotype heterozygotes to DNP conjugated to keyhole limpet hemocyanin (DNP-KLH; Tokuhisa, Black, Tsu, Ledbetter, Hayakawa, Gadus, and Herzenberg, unpublished observations). In this kind of study, sera are taken from mice 7–21 days after the adoptive transfer. The sera are tested on plates coated with DNP-BSA to avoid measurement of anti-KLH antibody. Four tandem assays are performed for each serum to allow individual measurements of Ig-1a, Ig-1b, Ig-4a, and Ig-4b anti-DNP antibody in the serum. Each serum is tested in duplicate, generally at each of two dilutions, for initial screening. Those test sera falling outside the valid range of the assay are then diluted appropriately and retested to obtain accurate measurements. Responses are expressed as a percentage of the antibody present in a standard serum prepared by pooling sera from a large number of responding (control) recipients in an adoptive transfer similar to those transfers from which the test recipients are drawn. (Other standards can be used, but we have found it is wise to match test and standard sera as closely as possible.)

A wide variety of specific second-step reagents can be used in these assays, including antiallotype, anti-idiotype, anti-isotype, and anti–light chain. The amount of labeled reagent required must be determined for each system but will usually be approximately 20000 cpm in a freshly labeled preparation (using the labeling techniques described in Section 18.2). Once established, the same concentration of labeled reagent should be maintained throughout its use, despite decay of radioactivity; this is helpful for comparing results obtained in different assays.

Polyvalent anti-Ig reagents composed of mixtures of specific reagents can also prove useful for RIA; however, these reagents (especially polyvalent anti-Ig in which the proportion of antibodies specific for different isotypes or allotypes is unknown) must be used with caution. One of the component antibodies in such a reagent may be limiting and thus fail to completely reveal a significant proportion of first-step antibody binding even though the overall second-step antibody concentration appears adequate. The presence of antibody reactive with IgM in these polyvalent anti-Ig second-step reagents may give disproportionately high values because IgM antibody is pentomeric and thus potentially binds five times as much radiolabel per mole of antibody.

One last caution: Solid-phase RIAs, like all antibody assays, cannot detect antibody whose avidity is below a certain level. Ignorance of the lower limit of avidities detectable in an assay (e.g., measurement of primary (low-avidity) antibody responses) can lead to falsely negative results.

In the anti-DNP assay performed as described here with DNP_{12}-BSA as coat antigen, we have found that primary responses to DNP-KLH on alum, with or without adjuvants, are only poorly detected. However, it is possible to extend the range of avidities detected by using DNP-BSA conjugates with higher hapten substitution ratios. Thus, coating with DNP_{19}-BSA or DNP_{42}-BSA allows detection of primary and similar low-avidity secondary responses, whereas coating with DNP_{5}-BSA and DNP_{12}-BSA allows detection of only the relatively high avidity components in a secondary response (see Table 18.2). Backgrounds are not substantially increased with the more highly conjugated antigens.

TABLE 18.2
Low-avidity anti-DNP antibody is revealed better by binding on plates coated with highly conjugated DNP-BSA

Source of α-DNP antibodies	Expected average avidity	α-DNP binding* (RIA plate coat)	
		DNP_{12}-BSA	DNP_{42}-BSA
In situ primary	low	2	16
Adoptive primary			
KLH 1° spleen	low	3	19
KLH 1° nylon T	low	0	6
Adoptive secondary			
2-week DNP 1° B (+ KLH 1° T)	low	13	34
8-week DNP 1° B (+ KLH 1° T)	high	150	150
DNP-KLH 1° spleen (assay standard)	high	100	108
In situ hyperimmune	high	360	350

* Binding expressed as units of α-DNP activity per ml of sample serum relative to the binding of the assay standard on DNP_{12}-BSA. Standard binding is arbitrarily set at 100 units/ml; thus a serum with 1 unit/ml has 1% of the α-DNP binding activity present in the standard.

The differences in the avidity of the antibody detected were established using a hapten-inhibition RIA. In this assay, ε-DNP-lysine in $\log_{10}$ dilution steps was preincubated for one hour with a constant dilution of antibody in a separate plate. A constant volume (20μl) of each of the incubated mixtures was then transferred to antigen-coated wells, and the assay was continued in the usual manner. Antibody dilutions were established to give a standard number of second-step (anti-Ig) counts bound for all samples in the absence of hapten inhibitor. Since the amount of second-step binding reflects the amount (μg) of first step bound, standardizing samples in this way allows comparison of avidities of different antibody samples for most sera without determination of the actual number of micrograms per milliliter of antibody in the serum.

This assay gives typical hapten inhibition curves (see Figure 18.1); however, the counts bound must be converted into units of antibody (see above) before plotting the curves, to correct for the nonlinearity of the original antibody titration.

As a routine screening procedure for high- and low-avidity antibody in responses, we have found that subtraction of units of antibody bound to DNP_{12}-BSA from units bound to DNP_{42}-BSA gives a reliable estimate of the low-avidity component in the sample.

The procedures described above for measuring low- and high-avidity antibody were developed by Takeshi Tokuhisa, Kyoko Hayakawa, Tim Gadus, Jeff Ledbetter, Sam Black, and the authors.

Figure 18.2 depicts a sample work sheet used to screen supernatants from hybridoma cell lines for antibody against antigens on cell surfaces (see Section 18.6) or antigen coated on Microtiter plates.

supernates

MATERIALS AND REAGENTS

As described in Section 18.3 (except for the deletion of myeloma proteins), with the following additions:

DNP-BSA, made as described by Iverson (1978): Adjust stock concentration to 10 mg/ml in PBS as determined by Bio-Rad protein assay (Bio-Rad Laboratories, #500-0001). Conjugation ratios are determined by the spectrophotometric method described by Iverson (1978). Aliquot DNP-BSA into small plastic tubes and store at −20°C. Dilute solution 1 : 100 in PBS to 0.1 mg/ml prior to use.

Normal mouse serum (NMS)

Anti-DNP serum for preparing standard curve (e.g., serum from an anti-DNP adoptive secondary response as described by Okumura et al. 1976)

PROCEDURE

1. Dilute DNP-BSA to 0.1 mg/ml in PBS and add 50 μl to an appropriate number of wells (all tests should be run in duplicate).
2. Cover plate with Parafilm and incubate for 1 hour at room temperature.
3. Dump plate and wash 3 times with RIA buffer. Dry plate after last wash by throwing plate face down onto absorbent paper.
4. Add 20 μl of appropriately diluted test or standard control serum to each well. Each assay should include a standard curve in dilutions (1 : 1000 to 1 : 32000, depending on the antiserum) of an adoptive secondary or other anti-DNP serum pool. Test sera should be diluted at least 50-fold (1 : 50) to avoid background problems. Wells with NMS (1 : 50) and RIA buffer should be included in the assay as negative controls.
5. Cover plates with Parafilm and incubate 1 hour at room temperature.
6. Dump plates and wash 3 times with RIA buffer. Dry after the last wash by throwing plate face down onto absorbent paper.
7. Add 20 μl of ^{125}I-labeled second-step reagent (10000 to 20000 cpm/well). (Note: Plate a 20 μl sample of the ^{125}I-labeled reagent in a counting tube at this time. Count this tube to determine the total cpm/well actually added in the assay.)
8. Cover plates with Parafilm and incubate 1 hour at room temperature.
9. Aspirate the radioactive supernatant and discard appropriately. Wash plates 3 times and dry after last wash.
10. Cut wells apart and count in gamma counter (as described in Introduction).
11. Plot standard curve on linear scale (counts vs μl of standard/well; see Figure 18.3). Generally, the curve will have an initial "linear" portion before flattening towards saturation. Whenever possible, read diluted test sera values from this linear portion since it gives the most accurate readings. (It is often wise to repeat tests on sera that do not test within the "linear" range of the assay, using a more diluted sample of each test serum, if highly accurate readings are desired.) Values read from the curve will indicate the microliter equivalent of the standard in the diluted test sample. Correct for dilution to obtain μl of standard equivalent/μl undiluted test serum. Results can also be expressed as a percent of the standard.

18.5 RADIOIMMUNE BLOCKING ASSAY: MEASUREMENT OF IMMUNOGLOBULIN LEVELS

The quantitation of immunoglobulin (Ig) levels in this assay is based on competition between bound and soluble Ig for radiolabeled antibody. The wells of a Microtiter plate are first coated with saturating amounts of antigen (e.g., Ig of a given class or allotype such as an Ig-1a myeloma protein). After excess coating antigen is removed by washing, a second volume of antigen is added to the wells. Some of the wells receive graded concentrations of the known standard, making it possible to construct a standard curve for reference; others receive the unknown test samples. Negative control wells receive no antigen (or receive antigen that does not cross-react with the coat antigen). All wells then receive ^{125}I-labeled antibody (e.g., anti–Ig-1a) at a fixed, limiting concentration. After incubation, plates are washed and the wells counted in a gamma scintillation counter.

Wells with no antigen added as competitor bind the maximum bindable fraction of the counts (antibody) added. Wells containing competitor antigen bind fewer counts because the soluble antigen binds some of the added counts so that those counts are not available for binding to the antigen on the well. (Complexes of soluble antigen and radiolabeled antibody apparently do not adhere to the precoated well.) A standard curve is drawn from the titration of the known standard. Amounts of antigen in the test samples are then calculated from this standard curve. Plotting the log of the amount of myeloma protein (or normal serum standard) against the number of counts bound generally yields a straight line, often over a thousandfold concentration range (depending on the labeled reagent used). The linearity of this curve makes it easy to interpolate to determine the quantity of the antigen in the test serum and to express this quantity in terms of milligrams per milliliter or a percentage of the normal serum standard.

MATERIALS AND REAGENTS

As described in Section 18.3, with the following additions:

Test serum
Negative control serum containing the wrong allotype

PROCEDURE

1. Dilute myeloma protein to 20 μg/ml in phosphate-buffered saline (PBS). Add 50 μl to an appropriate number of wells. Run all tests in duplicate.
2. Cover plates with Parafilm. Incubate for 1 hour at room temperature.
3. Dump the plate and wash twice with RIA buffer.
4. Refill the plate with RIA buffer. Leave this third wash on the plate for 1 hour at room temperature to allow any loosely bound protein to escape. Dump plate and dry.
5. Add 20 μl of standard myeloma protein solution (or normal mouse serum known to contain immunoglobulin of the appropriate allotype) to the appropriate number of wells. To obtain a standard curve, use serial dilutions of the original myeloma protein coat (starting at 20 μg/ml and decreasing to 0.02 μg/ml) or the normal mouse serum (starting at 1 : 10).
6. Add 20 μl of test serum or negative control serum to the other wells. Test serum dilutions of between 1 : 100 and 1 : 500 usually fall in the range of the standard curve. The negative control serum is tested at the same dilutions.
7. After adding all of the sera in step 5 and 6, add 20 μl of ^{125}I-labeled antibody reactive with the myeloma protein coated to the plate (40000 cpm/well). Incubate for 1 hour at room temperature.
8. Aspirate radioactive supernatant and discard appropriately. Wash plates 3 times and dry.
9. Cut wells apart and count in gamma counter (as described in Section 18.1)
10. Plot standard curve on semilog paper (counts vs log of blocking protein concentration; see Figure 18.4). Read amounts in test serum dilutions from standard curve. Correct for dilution to obtain mg/ml in undiluted test serum.

18.6 CELL BINDING ASSAY: CELL SURFACE ANTIGENS ON LYMPHOCYTES

Antibodies to cell surface antigens can be detected in a two-step assay in Microtiter plates by binding antibody to target cells and then detecting bound antibody with ^{125}I-labeled second-step reagents (e.g., antiallotype or staphylococcus protein A). This assay can also be used to determine the presence or absence of a cell surface determinant in a cell population or to determine the *average* amount of a surface antigen expressed on cells in a population. Because the procedure deals with a total population, it does not allow determination of the distribution of cells carrying different amounts of the surface antigen within the population. Thus, with this assay, a population of many cells each carrying a small amount of antigen cannot be distinguished from a population of a few cells carrying a large amount of antigen among many antigen-negative cells. Data on the distribution of cells carrying different amounts of surface antigen within a population require the use of methods in which cells are examined individually, for example, analysis with the fluorescence-activated cell sorter (FACS) as discussed by Herzenberg and Herzenberg (1978b).

Target cells (e.g., spleen; see Chapter 1 for general procedures for obtaining mouse cell suspensions)

Positive control antiserum, known to bind to target cells and detectable by the second-step reagent

^{125}I-labeled second-step reagent (e.g., anti-antibody or staphylococcus protein A; Section 18.2)

Phosphate-buffered saline (PBS; Appendix A.8)

RIA buffer, 5% FCS in PBS, 0.02% NaN_3: Prepare as described in Section 18.3, except add 5% FCS instead of BSA.

Microtiter plates, "U" bottom (Cooke Laboratory Products, 96-2311 plates, #220-24)

Microtest II plate lids (Falcon, #3041 or similar)

Microtiter mixer (Cooke Laboratory Products)

Plate holder, rubber (Cooke Laboratory Products, #220-26)

Pipetman, 20 μl and 200 μl (Rainin Instrument Co.)

Nitex monofilament screen cloth, 40 × 42 in. × 5 yd (TETKO, #HD-85)

Glass wool column: Fill a 3-ml disposal syringe barrel with approximately 0.75 ml glass wool for each spleen to be filtered. Incubate the column with RIA buffer for 15 minutes prior to use.

Heat lamp to dry Microtiter plates

Hot wire cutter (D. Lee)

Glass slides, 1 × 3 in., frosted ends

Gamma scintillation counter

Mishell 34-3029—Gal. 186

2

PROCEDURE

1. Remove target cell organs (e.g., spleen) from mice and make a single-cell suspension in RIA buffer by gently squeezing organs between frosted ends of 1 × 3-in. glass slides and passing cells through nylon monofilament cloth.
2. Centrifuge at 290 × g for 10 minutes (1000 rpm in an IEC centrifuge), wash cells with RIA buffer, and resuspend cells in 1.0 ml RIA buffer per spleen.
3. Remove dead cells by filtration through glass wool (Section 1.21). Apply the cell suspension to the column (avoiding a pressure head) and immediately follow with 10 ml of RIA buffer at room temperature. Collect all the effluent.
4. Centrifuge and resuspend as in step 2 (cell concentration will be approximately 1×10^8 cells/ml). Count cells and adjust 2×10^7 cells/ml.
5. Add 20 μl of the cell suspension to Microtiter wells (20 μl of 2×10^7 cells/ml = 4×10^5 cells/well). Tape rubber plate holders to the Microtiter plates to provide support.
6. Add 20 μl of the test solution (containing antibody appropriately diluted in RIA buffer) to the cell suspension in the wells. Mix plates thoroughly on a Cooke Microtiter mixer, cover the plates with lids, and incubate the plates for 30 minutes at 4°C. Mix the plates again and incubate for an additional 30 minutes.
7. After the second incubation, add 100 μl of RIA buffer to each well. Centrifuge plates at 290 × g for 3 minutes (1000 rpm in an IEC centrifuge with Cooke centrifuge carriers)
8. Aspirate supernatant with a blunt-end, 23-gauge needle attached to a vacuum. Add 200 μl of RIA buffer to each well. (Directing the buffer stream at the pellet facilitates resuspension.) Mix plates and check visually to see that the cells are thoroughly resuspended. Centrifuge plates at 290 × g for 3 minutes

9. Aspirate the supernatant. Add 50 μl of ^{125}I-labeled (10000–20000 cpm) second-step reagent. (Note: Place a 50-μl sample of the ^{125}I-labeled reagent in a counting tube at this time. This tube will be counted to determine the total cpm/well actually added in the assay.) Mix plates thoroughly. Incubate the plates for 30 minutes at 4°C, mix again, and incubate for an additional 30 minutes at 4°C.
10. Repeat steps 7 and 8, with an extra 200-μl cycle, to wash away the unbound radiolabeled reagent. Collect the first wash, containing the bulk of the radioactivity, into a special container for appropriate disposal.
11. After the last wash, aspirate the supernatant and dry the plates for about 5 minutes under a heat lamp.
12. Cut the wells apart, place wells in counting tubes, and count in a gamma scintillation counter.

COMMENTS

1. We routinely include 6 negative controls composed of RIA buffer plus target cells and 6 positive controls composed of an antibody known to bind to the target cells plus the target cells themselves. This positive control antibody also must be known to be detectable by the second-step (radiolabeled) reagent.
2. The second-step reagent must not, by itself, bind appreciably to the target cells. Otherwise, background values will be too high to assess binding of the first-step reagent. Thus, anti-IgM and anti–Ig light chain reagents cannot be used when spleen cells are used as targets because of the large amount of surface IgM and IgD on splenic B cells. Fortunately, very little surface IgG is present on these cells; therefore, it is possible to use specific anti-IgG second-step reagents without generating appreciable backgrounds.
3. Occasionally, cells appear (by eye) to disappear during the assay. Despite this occurrence we recommend that the assay be carried out to completion as the assay can still give interpretable values.
4. To make the cell pellet more visible, "carrier" erythrocytes can be added. Controls should then also include wells with the carrier cells alone.
5. Preliminary data suggest that antibodies to H-2, Ia, and other lymphocyte cell surface antigens are detectable in plate binding as well as cell binding assays. Plates are coated with isolated cell surface membranes prepared ans suspended in detergent-free aqueous buffers containing Mg^{2+}. Assays for antibody are then conducted as described in previous sections. Optimal protocols are currently under development (Dr. S. Qasim Mehdi, Department of Radiology; Frank Howard, Department of Chemistry; and Dr. Jeffrey Ledbetter, Department of Genetics; all at Stanford University).

d —

18.7 AUTOMATED PIPETTING DEVICE:

Assaying large numbers of samples (many plates per day) can be considerably facilitated by the use of automated pipetting devices; however, such devices must meet the standards of accuracy desired in the assay. In a rough survey of equipment available on the market in 1977, we were unable to find equipment that satisfied our needs. We therefore constructed the following device.

We ordered a special programmable pipetting apparatus* that had gauged peristaltic pump heads capable of handling up to 12 individual pipetting lines in parallel. We connected each of these lines to a plastic fitting milled to hold the standard plastic tips used on a Pipetman. The 12 fittings were then mounted in a "wand" so that they could be moved from plate to plate as one unit. The fittings were rigidly aligned in the wand such that the tips were centered over the 12 wells in one row of a Microtiter plate. Thus 12 samples (one row) could be simultaneously withdrawn or added to a plate (see Figure 18.5). Harleysville

* 12-Channel Programmable Micro-Pipetting Pickup and Delivery System, Innovative Medical Systems, Harlesville, PA. spelling correct?

For operation, a set of plastic tips are affixed to the fittings in the wand, the lines are seated in the peristaltic pumps, and the free ends are placed in a reservoir containing RIA diluent. The lines are then filled with RIA buffer and the bubbles removed if necessary. The apparatus is then ready for use. The diluent is maintained in the line and driven through before each new sample is pipetted to prevent carry-over between samples (i.e., tips are washed from within). The entire set-up procedure takes about five minutes.

To control the volume of fluid pipetted and the sequence of pickups, deliveries, and washes as samples are transferred from "supply" wells on one plate to "test" wells on another, the programmable pipetting apparatus (which in itself can control volume) is placed under the control of a microprocessor unit that can be programmed to repeat a complex series of steps, with each successive step being triggered by a foot pedal signal. We have written programs for the standard assay protocols in use in the laboratory. One such program, for example, allows duplicate samples to be pipetted from rows of supply wells with intervening washes in between rows; the program dictates: Take in 30 μl air (to separate sample from diluent in line), pick up 20 μl from supply wells, deliver 20 μl to test wells, pick up 20 μl from supply wells, deliver 20 μl to (duplicate) test wells, deliver 200 μl diluent to waste collection vessel (to wash), restart sequence.

Over a long period of use, we have found the accuracy of work with this apparatus equal to or greater than the accuracy of pipetting samples with a Pipetman if we change Pipetman tips between samples. If we merely rinse the Pipetman tips between samples, the accuracy of the "automatic" apparatus is greater because of occasional carry-over between samples transferred with a Pipetman.

We are currently building a fully automated system based on the above principles, where supply and test plates are shuttled into position under the wand and the wand raised and lowered into the wells as required. In this version of the system, the programmable pipetting control system has been eliminated and the microprocessor is used to directly control the peristaltic pumps (i.e., the volumes delivered).

Personnel involved in designing the manual and automated systems include: William Harlow, Tom Nozaki, Jr., Wayne Moore, David Parks, and Leonore Herzenberg, with a sizable assist from facilities in our laboratory operated under the direction of Richard Sweet and Leonard Herzenberg.

REFERENCES

Herzenberg, L. A., and L. A. Herzenberg. 1978a. Mouse immunoglobulin allotypes: Description and specific methodology. In D. M. Weir (Ed.), *Handbook of Experimental Immunology*, third edition. Blackwell Scientific Publications, Oxford.

Herzenberg, L. A., and L. A. Herzenberg. 1978b. Analysis and separation using the fluorescence-activated cell sorter (FACS). In D. M. Weir (Ed.), *Handbook of Experimental Immunology*, third edition. Blackwell Scientific Publications, Oxford.

Iverson, G. M. 1978. Assay methods for antigen-mediated cell cooperation. In D. M. Weir (Ed.), *Handbook of Experimental Immunology*, third edition. Blackwell Scientific Publications, Oxford.

Miles, L. E. M., and C. N. Hales. 1968. Labelled antibodies and immunological assay systems. *Nature* 219:186.

Okumra, K., L. A. Herzenberg, D. B. Murphy, H. O. McDevitt, and L. A. Herzenberg. 1976. Selective expression of *H-2* (*I*-region) loci controlling determinants on helper and suppressor T lymphocytes. *J. Exp. Med.* 144:685.

Okumura
u
+ transpose

Fig 18.4A
18.4-a

distributions of low and high avidity anti-DNP serum pools: DNP42-BSA RIA plate coat.

Avidity of anti-DNP antibodies from Ig-5b$^+$ or Ig-5b$^-$ B cell response.

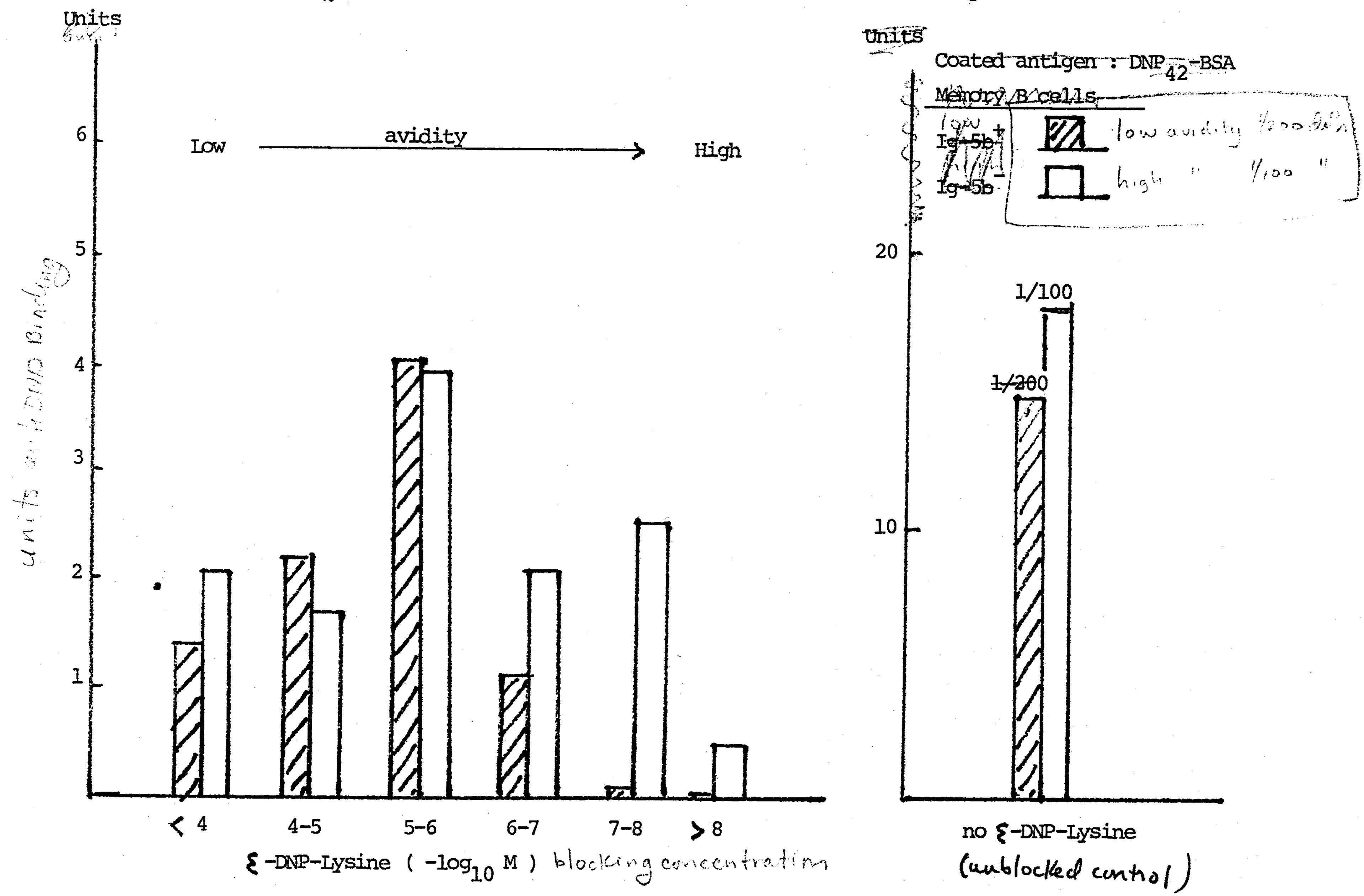


Table 18.4

LOW AVIDITY ANTI-DNP ANTIBODY IS REVEALED BETTER BY BINDING ON PLATES COATED WITH HIGHLY CONJUGATED DNP-BSA

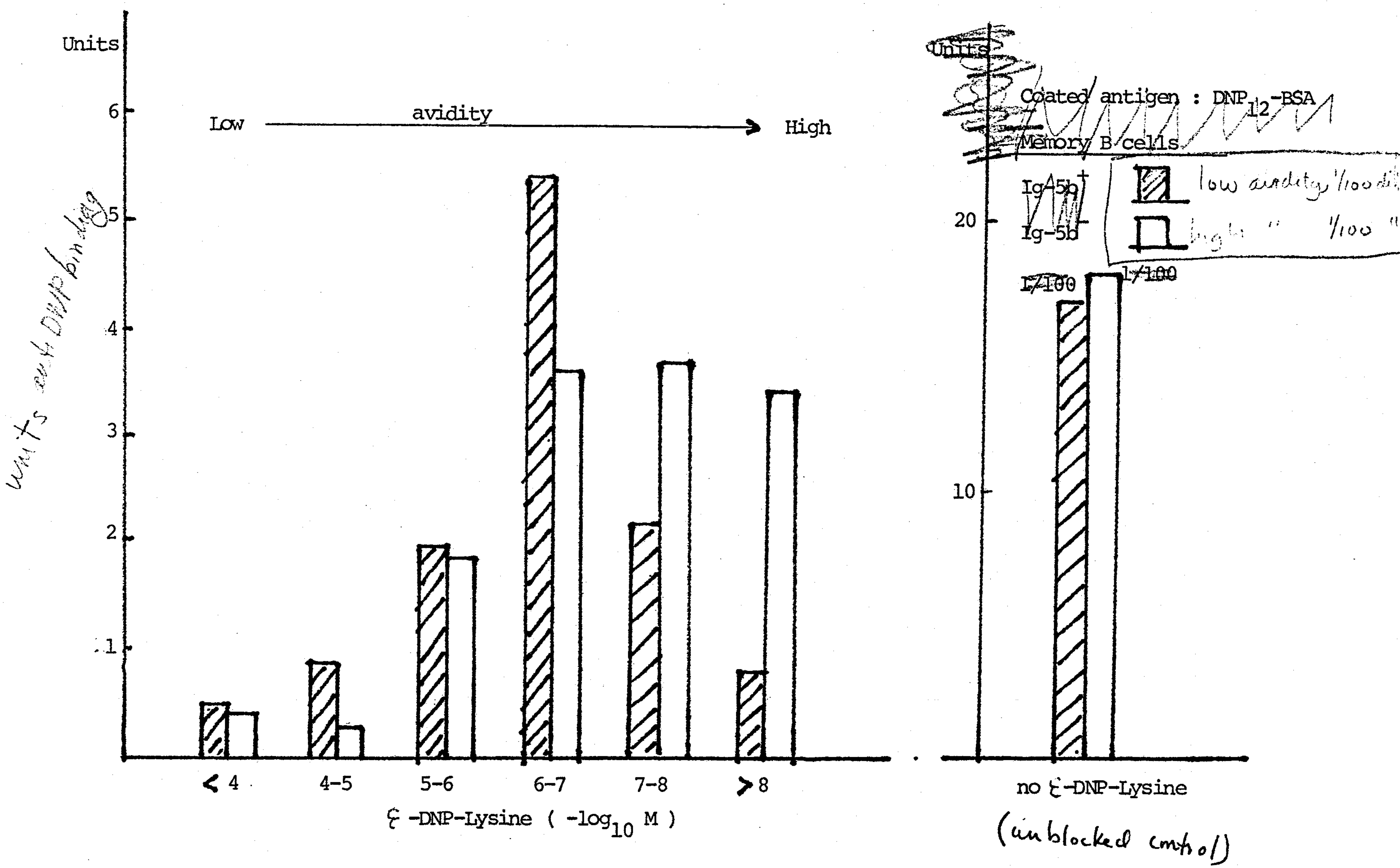
Source of α-DNP Antibodies	Expected Average Avidity	α-DNP Binding* (RIA plate coat) $DNP_{12}BSA$	$DNP_{42}BSA$
In situ primary	low	2	16
Adoptive primary			
KLH 1^o spleen	"	3	19
KLH 1^o nylon T	"	0	6
Adoptive secondary			
2 wk DNP 1^o B (+ KLH 1^oT)	"	13	34
8 wk " "	high	150	150
DNP-KLH 1^o spleen (assay standard)	"	100	108
In situ hyperimmune	"	360	350

*Binding expressed as units α-DNP activity per ml sample serum relative to the binding of the assay standard on $DNP_{12}BSA$. Standard binding is arbitrarily set at 100 units/ml; thus a serum with 1 unit per ml has 1% of the α-DNP binding activity present in the standard.

18.4 - b

Distributions of low and high avidity anti DNP serum pools: DNP15-BSA on plate coat;

Avidity of anti-DNP antibodies from Ig-5b^{+} or Ig-5b^{-} B cell response

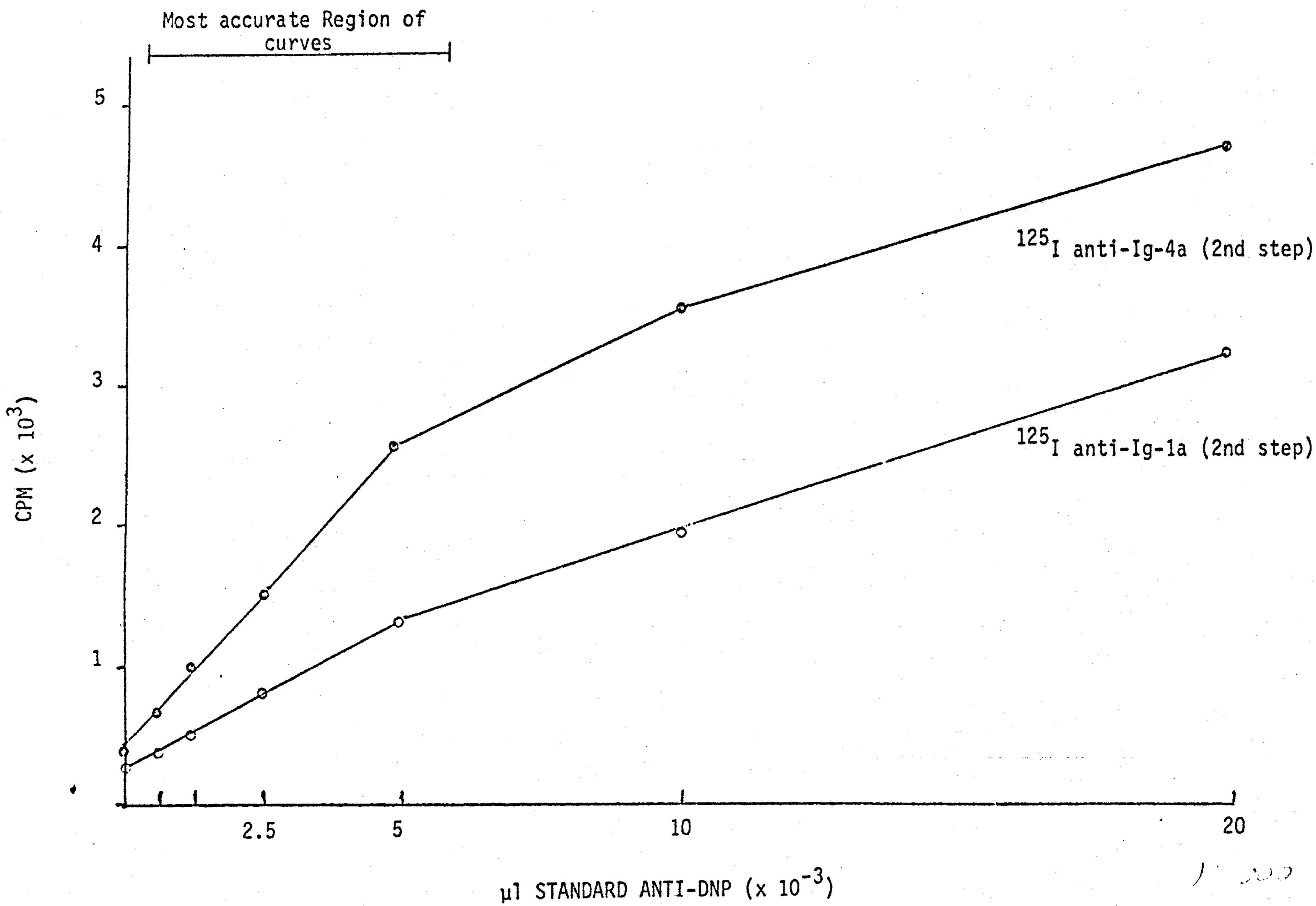


LEGEND FOR FIGURE

Figure 18.4. Avidity Distributions of Anti-DNP Antisera Measured on DNP_{12}-BSA and DNP_{42}-BSA RIA Plate Coats.

Bars in the figure represent units of anti-DNP antibody not blocked by the lower ε-DNP-lysine concentration but blocked by the higher. (For unit definition, see legent for Table 18.4). Low avidity pool contained 17 units anti-DNP activity measured on DNP_{12}-BSA and 30 units measured on DNP_{42}-BSA; high avidity pool contained 18 units anti-DNP activity on either coat. Antibody dilutions used were adjusted to equalize binding between low and high avidity pools for each plate coat.

Fig 18.4 c
18.4-c



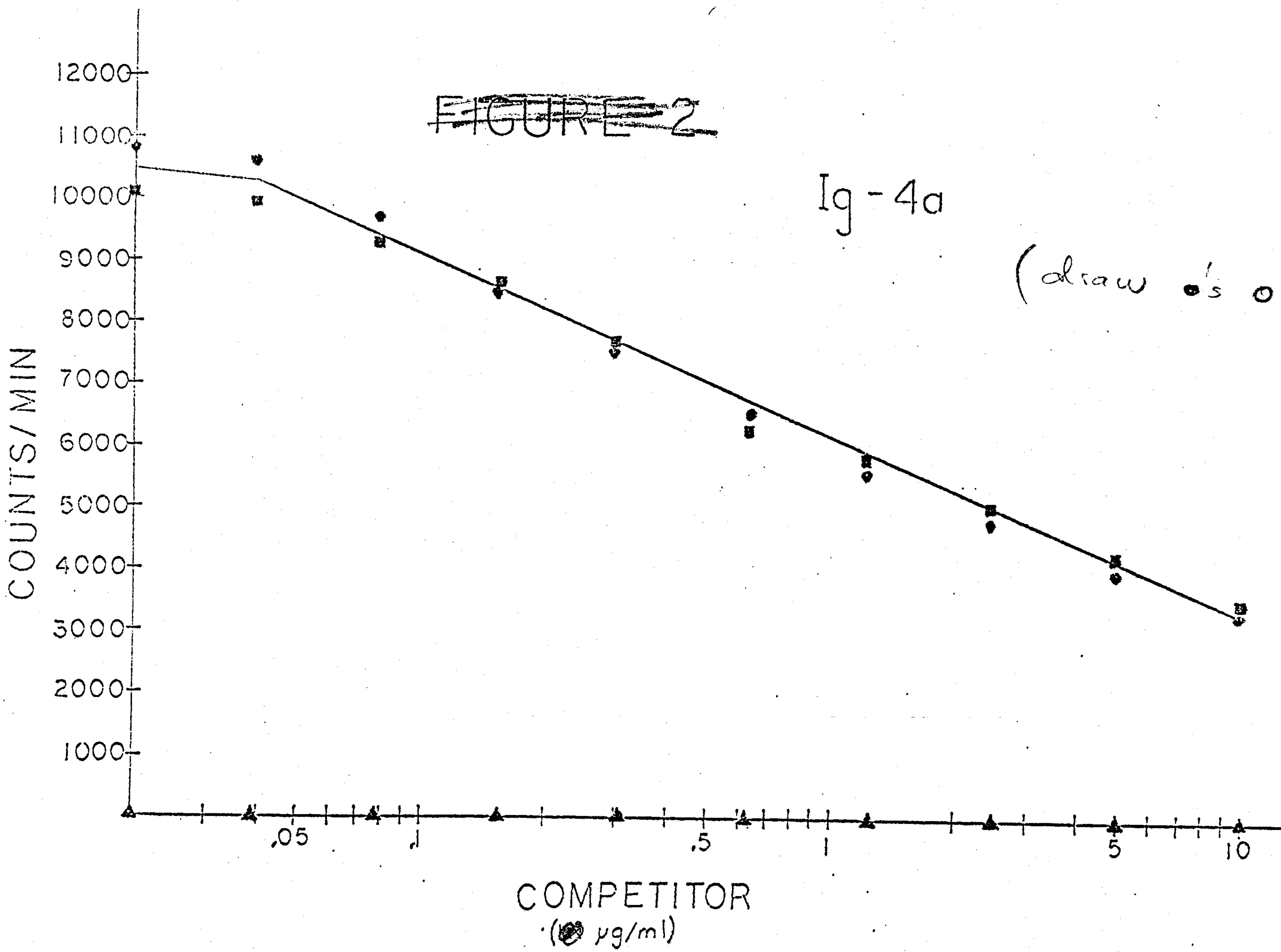
DS TO FIGURES

~~Figure 1. Protocol Planning and Data Recording Sheet: Hybridoma Supers~~

18.4c

Figure ~~2~~. Anti-DNP Standard Curves with Different Anti-allotype second-step Regents. 1st step: 20 µl standard anti-DNP. 2nd step: 20 µl ^{125}I-anti allotype. Plate coat: 50 µl DNP_{12}-BSA at 0.1 mg/ml.

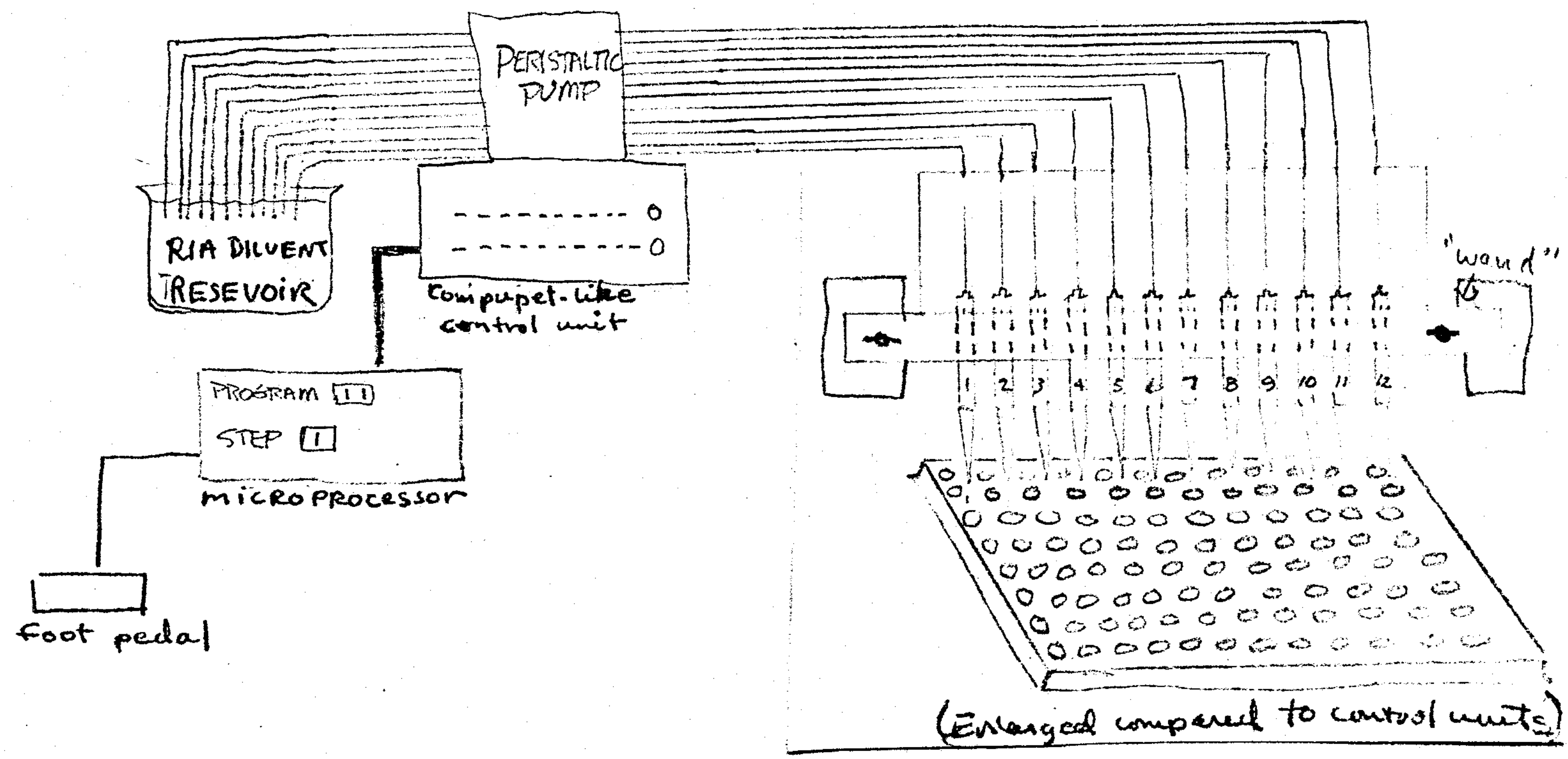
Figure 18: 5


~~FIGURE 2~~
Ig-4a
(draw •'s only)
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
COUNTS/MIN
.05
.1
.5
1
5
10
COMPETITOR
(µg/ml)

Legend for Figure 18.5

Standard Curve for the ~~Plate Binding Assay~~ RIA blocking assay. When counts per minute are plotted against the logarithm of the concentration of antigen added to the wells to compete with the plate coat, a straight line is obtained. In this assay, MOPC-21 was used as the plate coat and the competitor. ~~The standard curve was run twice (• first run, ■ second run) in duplicate in the assay.~~

Fig 18



PERISTALTIC PUMP
RIA DILUENT RESEVOIR
Compupet-like control unit
PROGRAM
STEP
microprocessor
foot pedal
"wand"
1
2
3
4
5
6
7
8
9
10
11
12
(Enlarged compared to control units)

Expt. # ____________ Hybr.-Plate No. ____________ Date ____________

Cell type ____________ Strain ____________ Cells/well ____________

Coat antigen ____________; Super. vol/well ____________ Super. date ____________

Labeled reagent ____________ ID # ____________ cpm/vol. added ____________

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

A												
B												
C												
D												
E												
F												
G												
H												

Expt. # ____________ Hybr.-Plate No. ____________ Date ____________

Cell type ____________ Strain ____________ Cells/well ____________

Coat antigen ____________; Super. vol/well ____________ Super. date ____________

Labeled reagent ____________ ID # ____________ cpm/vol. added ____________