Radioimmunoassay on Polycarbonate Membranes: a Sensitive and Simplified Method for the Detection and Quantitation of Antibody

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A new radioimmunoassay technique is described in which a sampling manifold and polycarbonate filter membranes are used. Antigen-antibody reactions take place on the membranes. The manifold permits the simultaneous running of 30 samples and a rapid wash technique in which the washing solution is introduced into the manifold wells and suctioned off from below. The polycarbonate membrane offers a distinct advantage over the cellulose acetate in that nonspecific protein adsorption is substantially reduced. Antigens employed are whole bacteria or soluble antigen coupled to Sepharose beads and are layered over the membrane surface. A radiolabeled antibody is used to measure the primary antigen-antibody interaction. The sensitivity of the procedure is equal to that of fluorescent antibody tests.

The coupling of antibody or antigen to a solid phase component for ultimate use in radioimmunoassay has been adapted to several scientific disciplines. Numerous workers have used this technique with individual modifications (12). Wide et al. (11) have been particularly inventive in the field of allergic investigation with their development of the radioimmunosorbent technique (RIST) for quantitation of total immunoglobulin E and the subsequent description of the radioallergosorbent technique (RAST) for characterizing specific immunoglobulin E (IgE). In the latter technique, soluble antigen is coupled to a solid particle surface. This is then reacted with the serum of an allergic individual, thereby binding any IgE directed against the allergen to the solid phase. The allergen-IgE complex is then allowed to interact with radiolabeled anti-IgE, which serves as the indicator of the first stage antigen-antibody reaction.

The Swedish group (2) has modified the original RAST description by coupling antigens to standard cellulose acetate filter membranes, instead of the more usual beads, before the incubation with patient's serum. Unfortunately, attempts to use the RAST assay for detection of specific immunoglobulins other than IgE have been hampered by the high nonspecific adherence of normal serum proteins to the beads and membranes (R. Evans, RAST Technique, 29th Annu. Meet. Amer. Acad. Allerg., Washington, D.C.).

A radiolabeled antibody offers distinct advantages over a fluorescein labeled antibody as an indicator of a primary antigen-antibody interaction. As pointed out by Hutchinson and Ziegler (6), recording radioactive counts is actually quantitative and contrasts with the semi-quantitative read-out of the indirect fluorescent antibody procedure. In addition, because of the difficulties associated with the development of excitation scanners, the automation potential of fluorescence work is not as great as that of the radioassays. These workers capitalized on the advantages of radioassay in their recently reported work. Bacterial and viral antigens affixed to cover slips were allowed to react with patient's serum. A radiolabeled antibody was then employed to detect any antigen-antibody associations.

In this paper we describe a new technique which borrows from principles illustrated by the above techniques. Whole bacteria or soluble antigens coupled to beads are layered over the surface of filter membranes held in the sampling manifold. The manifold permits the simultaneous filtering of as many as 30 10-ml samples and facilitates the use of an easy wash technique in which the wash solution is introduced into the individual wells and suctioned off from below. The antigens are physically held on the membranes during filtration because they are larger than the pore dimensions of the membranes. The antisera are then added sequentially, and antigen-antibody reactions

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occur on the membrane surface. The indicator antibody is radiolabeled.

Polycarbonate membranes were used throughout this work. Nonspecific protein adherence to the polycarbonate membrane was substantially lower than the adherence to cellulose acetate membranes. Because of this feature, a RAST-like assay for detection of antibodies other than IgE is now possible.

MATERIALS AND METHODS

Filters and manifold. A no. 3025 sampling manifold was used (Millipore Corporation, Bedford, Mass.) as well as Millipore (R) cellulose acetate filter membranes, 0.45 μm pore size and 25 mm diameter. Polycarbonate filters were obtained from the General Electric Corporation, Vallecitos Road, Pleasanton, Calif.; these were of 0.4 μm pore size and 25 mm in diameter. Two experiments were done with filters of pore size 0.5 μm.

Preparation of antigens. A 16-μg amount of rabbit albumin (RSA) (Pentex Corporation, Kanakakee, Ill.) was coupled to 20 ml of Sepharose 4B (Pharmacia Labs, Inc., Piscataway, N.J.) by the cyanogen bromide technique (3). Type I gonococci (8) were obtained from agar plates with standard harvesting techniques. The diameter of the gonococcus is 0.6 to 1 μm. After formalization (1% solution), the gonococci were suspended in phosphate-buffered saline (pH 7.2) containing 0.05% sodium azide, to a standard density of 0.3 (known to equal 10⁷ colony-forming units per cm²). A Beckman spectrophotometer (Beckman, Fullerton, Calif.) was used at a wave length of 530 nm.

Preparation of antisera. The goat antibody was isolated by diethylaminoethyl (DEAE)-cellulose chromatography from goat anti-rabbit serum that was produced at the Center for Disease Control (CDC). The rabbit anti-goat antibody was obtained from a rabbit immunized at the CDC. This antibody also was gamma G isolated by DEAE-cellulose chromatography and had anti-whole serum activity. The anti-human gamma globulin was prepared by passing rabbit anti-whole serum through an immunoabsorbent column prepared by coupling gamma globulin (Cohn fraction II) to Sepharose 4B particles with CNBr (3) and eluting with 0.1 M glycine-hydrochloride (pH 2.6). The 125I labeling was performed by the McConahey and Dixon (9) modification of the method of Hunter and Greenwood (5). The radiolabeled antibodies were used in the experiments at 1:1,000 dilutions, which (depending on the lot) added approximately 40,000 counts/min per 0.1 ml to the experimental material. Dilutions of the goat anti-rabbit and rabbit anti-goat antibody were made with the same phosphate-buffered saline solution used for washing (pH 7.2) with added 0.2% bovine serum albumin (BSA) and 0.05% sodium azide. The antigonococcal sera were obtained from patients seen at the DeKalb County Veneral Disease Clinic. The source of the high titer serum was an infected female patient with a high titer (1:2,560) of anti-gonococcal antibodies in a hemagglutination system. Control Nun sera were obtained from the CDC veneral disease serum bank. Goat IgG was obtained as a 7S cut of normal goat serum from a Sephadex G-200 column.

Experimental protocol. The washing solution was phosphate-buffered saline (pH 7.2) with 0.2% BSA and 5.0% Tween-20 (Fisher Scientific, Fairlawn, N.J.). The validity and reliability of the experimental system were first tested in plastic capped tubes (12 by 75 mm) (Falcon Inc., no. 2058, Oxnard, Calif.). Serum samples were refrigerated at 4 C. All centrifugation was performed at room temperature for 5 min at 1,000 x g. Specimens were incubated at 37 C for 1.5 h in the preliminary test tube experiments. A humidity chamber for the incubations was provided by placing a damp cloth towel over the top plate and, therefore, over the mouths of the individual wells of the manifold. A plastic cover was provided to maintain the humidity during the incubation. Sera were pipetted with a 100 μl Eppendorf pipette (Intertex Corporation, Waltham, Mass.). Gamma counting was performed in a Baird atomic counter (Baird Atomic, Cambridge, Mass.). Membranes containing samples were folded and inserted into disposable glass or plastic tubes (12 by 75 mm) and then placed into carrier tubes for the counting. The maximal amount of 125I antibody to be added in the gonococcal system was determined in experiments designed to saturate sites in the higher titer serum without increasing the backgrounds. The quantitative precipitin (7) test was performed on the goat anti-rabbit albumin antiserum to assess how much antibody was in the test serum. By appropriate dilutions of this goat anti-rabbit albumin antiserum, a limit to the detection properties of our RSA-bead system could be defined; this determined limit could then be converted to an exact quantitation of the amount of antibody in the serum, and some evaluation of sensitivity of the method could be made.

Procedure. Preliminary experiments performed in test tubes demonstrated activity of the goat anti-rabbit albumin and of the rabbit anti-goat globulin; similarly, activity was demonstrated for the antigonococcal serum and the immunoabsorbed rabbit anti-human gamma globulin. The reagents were then tested in the membrane filter system.

The antigen preparations, either 0.2 to 0.4 ml of type I gonococci or 0.1 ml of the RSA-bead complex, were pipetted onto the membranes in a sampling manifold and were allowed to disperse. These amounts were determined through several experiments designed to assess the capacity of the membranes to hold the particle and still permit adequate speed of washing. At this point, 0.1 ml of the antiserum (0.2 ml in some gonococcal experiments) was pipetted directly onto the particles. The manifold with membranes, particles, and antiserum was then incubated at 37 C for 30 min. The filter arm was left open to ambient air. After the incubation period the membranes with gonococci were washed twice with 10 ml of wash solution introduced with a 10-ml Cornwall syringe: in this sequence, the wash solution was introduced into all experimental wells and suction was applied so that all wells would drain simultaneously.
ously. In the RSA bead system, four 10-ml washes were necessary to wash the beads adequately. The filter arm was then disconnected from the suction tubing and the valve was again opened to ambient air. After these washes, 0.1 to 0.3 ml of the radiolabeled antiserum was added in the gonococcal test system, and 0.1 ml was added to the bead system. The quantities of serum and radioactive antiserum added were determined, in the case of the gonococcal work, through multiple preliminary experiments designed to optimize the system. Such studies were not carried out with the RSA-bead system. The humidification was again provided, and the membranes were incubated a second time for 30 min at 37 C. After the second incubation, the membranes were washed again exactly as described above. The membranes were then carefully lifted to a paper towel with forceps and a cotton-tipped applicator. At this point they were folded into a compact size and placed in glass tubes to be counted for 5 min or, in rare instances, for only 1 min. The final size or geometry of the folded filter membranes varied, but this did not appear to interfere with the reproducibility of counting. Duplicate membranes were used in the experiments, and the results were reported as an average of the two experimental membranes minus the average of the two background (or control) membranes.

RESULTS

Counts in one table of data should not be compared with those of another table. The results reported herein were tallied over several months of experimentation and with different reagent batches, and no correction was made for isotope decay. Results reported within each section are, however, from the same experimental period. In certain tables the counts have been rounded off to the nearest thousand for ease of comparison.

In early experiments, the cellulose acetate membranes were compared with the polycarbonate membranes. This comparison established that the experimental and radiolabeled antisera were absorbed prohibitively onto the cellulose acetate membranes. The background counts were extremely high, and the experimental reactive counts could not be separated from the background. The nonspecific adherence to the polycarbonate membranes was substantially lower, and these membranes were, therefore, employed exclusively for the remainder of the experimental period.

To provide maximum discrimination between experimental and background samples, numerous experiments were carried out. They were designed to increase the reactive counts or to decrease the background. Varying the incubation periods or the number of washes did not greatly alter results. On the other hand, adjustments in the composition of the wash solution did contribute to the lowering of the background counts (2,400 counts/min with 0.5% Tween, and 1,200 counts/min with 5% Tween) on the polycarbonate membranes. The final wash solution contained 5% Tween 20. Other washing reagents were tried—including guanine, urea, and different concentrations of BSA, Tween 20, and Tween 80. Neither presoaking the membranes for varying lengths of time in different wash solutions nor the provision of a 3- to 5-ml reservoir above the filters as a milieu for antigen-antibody interaction was effective in enhancing the system. Similarly, placing the entire manifold on a rocker in the incubator did not augment the antigen-antibody interactions.

Table 1 demonstrates the method used to determine the optimal quantities of radiolabeled antiserum to be added to the membranes.

As can be seen from the data in Tables 2 and 3, definite antigen-antibody interaction occurs on the polycarbonate membranes in both the bacterial and the soluble antigen-bead systems. The reactive counts recorded are well in excess of any nonspecific protein interactions (represented by employing 6% BSA in place of the antiserum) and nonspecific protein adherence to the beads, the bacteria, the membranes or any combination of these factors. Tables 4 and 5 indicate that the day-to-day reproducibility of the experiments was good and that the variability between duplicates was minimal. The RSA-bead system was more reproducible than was the gonococcal system, as will be discussed below. The 1:16 serum dilution (Table 2) was used in a number of experiments designed to separate those patients with cross-reactive group antibodies from those with true anti-gonococcal antibodies.

Table 1. Results obtained for different amounts of $^{131}I$ antiserum in the gonococcal system

| Gonococci (ml) | High titer anti-gonococcal antiserum (ml) | Rabbit antihuman $^{131}I$ (ml) | Counts/min$^a$ |
|----------------|------------------------------------------|---------------------------------|----------------|
| 0.4            | 0.1                                      | 0.1                             | a. 4,029$^b$   |
| 0.4            | 0.1                                      | 0.2                             | b. 4,371      |
| 0.4            | 0.1                                      | 0.3                             | a. 6,958      |
| 0.4            | 0.1                                      | 0.4                             | b. 7,591      |
| 0.4            | 0.1                                      | 0.4                             | a. 10,791     |
| 0.4            | 0.1                                      | 0.4                             | b. 11,041     |
| 0.4            | 0.1                                      | 0.4                             | a. 10,865$^c$ |
| 0.4            | 0.1                                      | 0.4                             | b. 13,761     |

$^a$ Background excluded.
$^b$ Both a and b represent results of experiments run on two successive days.
$^c$ Backgrounds rose to high of 3,200 counts/min.
The quantitative precipitin test was performed on the goat anti-rabbit albumin antiserum. This antiserum was a gamma G fraction and contained 900 µg of antibody per ml. The maximal dilution of this anti-serum which allowed distinction between the immunized goat and either the normal controls or back-ground was 1:16. This meant that in 1.0 ml of the stock antiserum 56 µg of antibody could be detected. Therefore, in 0.1 ml, a minimum of 5.6 µg is detectable. This is comparable to the sensitivity of the indirect fluorescent antibody test as reported by Atwood (1) (6 µg of IgG detected in the FTA-ABS test).

**DISCUSSION**

New uses for membrane filters have been fashioned recently at a prodigious rate. Cellulose acetate membranes have been used in a chamber similar to ours for lymphocyte trans-

**TABLE 2. Results obtained with different antisera**

| Gonococci (ml) | Antiserum (ml) | Rabbit anti-human $^{125}$I (ml) | Counts/min* |
|----------------|----------------|----------------------------------|-------------|
| 0.2            | 0.2 of high titer anti-gonococcal antiserum | 0.1 | 6,000 |
| 0.2 of PBS     | 0.2 of high titer anti-gonococcal antiserum | 0.1 | 1,800 |
| 0.2            | 0.2 PBS         | 0.1 | 1,600 |
| 0.2            | 0.2 of 6% BSA   | 0.1 | 400  |
| 0.4            | 0.1 of 1:16 concentration of high titer anti-gonococcal antiserum | 0.1 | 400  |
| 0.4            | 0.1 of 1:16 concentration of Nun’s serum | 0.1 | 6,000 |
| 0.4 of PBS     | 0.1 of 1:16 concentration of high titer anti-gonococcal antiserum | 0.1 | 1,800 |
| 0.4            | 0.1 of 1:16 concentration of another Nun serum | 0.1 | 2,800 |
| 0.4            | 0.1 of 1:16 concentration of serum from female patient with gonorrhea | 0.1 | 2,760 |

*Background excluded.

**TABLE 3. Results obtained using goat anti-rabbit albumin, $^{125}$I antisemirum, immune goat serum (gamma G fraction), normal goat IgG, and normal goat whole serum and appropriate background controls**

| Beads (ml) | Antiserum (ml) | Rabbit anti-goat $^{125}$I (ml) | Counts/min* |
|------------|----------------|----------------------------------|-------------|
| 0.1        | 0.1 of goat anti-RSA | 0.1 | 15,000 |
| 0.1        | 0.1 of normal goat | 0.1 | 1,400  |
| 0.1        | 0.1 of normal goat serum | 0.1 | 2,600  |
| 0.1        | 0.1 of goat anti-RSA | 0.1 | 2,600  |
| 0.1        | 0.1 of PBS         | 0.1 | 1,600  |
| 0.1        | 0.1 of goat anti-RSA | 0.1 | 2,200  |
| 0.04        | 0.1 of PBS         | 0.1 | 460    |
| 0.1        | 0.1 of normal goat | 0.1 | 2,000  |
| 0.1        | 0.1 normal goat serum | 0.1 | 2,000  |

*Background excluded.

**TABLE 4. Results obtained in experiments performed on two successive days**

| Gonococci (ml) | Antiserum (ml) | Rabbit anti-human $^{125}$I (ml) | Counts/min* |
|---------------|----------------|----------------------------------|-------------|
| I. 0.4        | 0.1 of 1:16 concentration high titer anti-gonococcal antiserum | 0.1 | 3,960 |
| II. 0.4       | 0.1 of 1:16 concentration high titer anti-gonococcal antiserum | 0.1 | 4,031 |

**TABLE 5. Results with duplicate samples in RSA-bead system**

| Beads (ml) | Goat anti-rabbit albumin antiserum (ml) | Rabbit anti-serum $^{125}$I (ml) | Counts/min* |
|------------|----------------------------------------|---------------------------------|-------------|
| 0.1        | 0.1 concentrated                        | 0.1 | 14,561 |
| 0.1        | 0.1 of 1:2 dilution                     | 0.1 | 15,867 |
| 0.1        | 0.1 of 1:4 dilution                     | 0.1 | 13,599 |
| 0.1        | 0.1 of 1:8 dilution                     | 0.1 | 8,784  |
| 0.1        | 0.1 of 1:16 dilution                    | 0.1 | 5,270  |
| 0.1        | 0.1 of 1:16 dilution                    | 0.1 | 4,167  |

*Background included.
formation work (10) and directly in a RAST assay. Attempts to employ a RAST-like assay for the detection of specific antibodies, other than IgE, have failed, and this has been attributed to the high nonspecific adherence of normal serum proteins to the membranes (12). The polycarbonate membrane has been adopted as a tool to separate T and B lymphocytes (4).

The polycarbonate membrane has several advantages over the cellulose acetate membrane for serologic work. It is a neutrally charged polymer with discrete unidimensional holes, whereas the interlacing meshwork of a cellulose acetate membrane presents a much greater surface area to a solution and offers a sponging effect. The polycarbonate membrane is also harder than the cellulose acetate membrane and is less likely to tear with manipulations such as folding. When soluble antigens coupled to beads are used (since no way is yet known to link soluble antigens chemically to the polycarbonate backbone), a RAST-like assay can now be performed on the surface of polycarbonate membranes. Whole bacteria can also be used as antigens on the membranes, in a system which is analogous to the indirect fluorescent antibody procedure but which capitalizes on the advantages of a radiolabeled antibody.

This work began as an attempt to develop a better serologic test for the diagnosis of gonorrhea. Despite numerous manipulations of the sera, there continued to be considerable overlap in the results obtained with patient's serum and the control. Therefore, these data should not be construed to imply that a workable test for the diagnosis of gonorrhea evolved from this study.

Additional points should be made. In the RSA-bead system, the “immune” serum was represented by a gamma G fraction, and the radiolabeled antiserum had anti-whole serum activity. Since there will be a certain small amount of protein adhering nonspecifically to the membranes when whole serum is used (in routine serology), it is suggested that an immunoadsorbed radiolabeled antiserum be employed for detection as in the gonococcal data (i.e., anti-total gamma globulin, or anti-IgG, IgM or IgA). This will reduce confusing background counts that would result if the radiolabeled antiserum had anti-whole serum activity.

Also, in the bacterial work, a large supply of the gonococci were prepared and standardized to the desired optical density, and this was used as a pool for future experiments. The optical density changed, however, as time elapsed, and this was attributed to either autoagglutination of the bacteria or to lysis. Since the quantity of beads or bacteria placed on the membranes is critical to the reproducibility of the system, optical densities should be rechecked frequently. Keeping the membranes with the beads or the bacteria moist throughout the experimental periods was also important to maximize the ultimate counts and to obtain good reproducibility.

It has, therefore, been demonstrated that antigen-antibody reactions can occur on the surface of polycarbonate membranes, and that a radiolabeled antibody can be used to detect this. A large number of tests can be done simultaneously, and washing steps are simplified by a sampling manifold. Substantially less nonspecific protein adheres to polycarbonate membranes than to cellulose acetate membranes, and this facilitates the successful measurement of specific antibody other than IgE in a RAST-like assay. This new technique is equivalent in sensitivity to the indirect fluorescent antibody tests.

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