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Comparison of Direct and Indirect Solid-Phase Microradioimmunoassays for the Detection of Viral Antigens and Antiviral Antibody

JOEL D. ROSENTHAL, KOZABURO HAYASHI, AND ABNER L. NOTKINS

Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20014

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Viral antigens were fixed to the surface of microtiter wells, and serial dilutions of antiviral antibody were added. The amount of antiviral antibody bound to viral antigens was determined by measuring the extent to which the antiviral antibody either inhibited the specific binding of ^{125}I-labeled antiviral immunoglobulin G (IgG) (direct technique) or enhanced the specific binding of ^{125}I-labeled anti-IgG (indirect technique). Immune complexes composed of viral antigens and antiviral antibody (human) could be detected by the binding of ^{125}I-labeled rheumatoid factor. Specific binding was influenced by the concentration of protein in the diluents used during the different steps of the procedure. A high concentration of protein in the diluent used with the viral antigens decreased specific binding, whereas a high concentration of protein in the diluent used with ^{125}I-labeled anti-IgG increased specific binding by decreasing nonspecific attachment of the labeled anti-IgG. Under the conditions employed, the titer of a given antiviral serum was several hundredfold greater by the indirect than by the direct technique.

Radioimmunoassays (RIA) have been widely used in the study of a variety of biological problems (20). However, the application of these techniques to experimental and diagnostic viral serology has been slow. Radioimmunoprecipitation (RIP) procedures have received the most attention (1, 4, 6, 7, 9, 12, 13, 17, 21, 24, 25, 28, 29). Generally, labeled virus is incubated with dilutions of antiviral antibody and precipitated with anti-immunoglobulin. The titer of antiviral antibody is determined by measuring the amount of radiolabeled virus that has been precipitated. Recently, solid-phase assays, based on either the attachment of radiolabeled antibody to viral antigens or the inhibition of attachment of radiolabeled virus to antibody-coated tubes (5, 10, 11, 15, 17, 22, 26; K. Hayashi, D. Lodmell, J. Rosenthal, and A. L. Notkins, J. Immunol., in press; J. Rosenthal, K. Hayashi, and A. L. Notkins, J. Gen. Virol, in press) have been used to detect viral antigens and measure antiviral antibody. The potential advantages of solid-phase over RIP assays and the use of radiolabeled antibody over radiolabeled virus are the greater speed in processing large numbers of samples and the greater availability and ease in labeling antibody. Solid-phase assays also can be miniaturized, and recently we described a technically simple and rapid indirect solid-phase micro-RIA for measuring antiviral antibody based on the attachment of ^{125}I-labeled anti-immunoglobulin to antiviral antibody (26). In the present report we enumerate some of the factors which affect the sensitivity of this indirect technique, describe a direct inhibition assay which employs ^{125}I-labeled antiviral IgG, and show how the indirect technique can be used to measure viral antigens.

MATERIALS AND METHODS

Tissue culture. Primary rabbit kidney cells (PRK) were prepared and maintained as previously described (14). Growth medium consisted of Eagle minimal essential medium with 0.5% lactalbumin hydrolysate, 10% heat-inactivated calf serum, and antibiotics (100 U of penicillin per ml, 50 μg of neomycin per ml, 100 U of mycostatin per ml, and 100 U of streptomycin per ml).

Preparation of viral and control antigens. Monolayers of PRK cells were infected with herpes simplex virus (HSV) (type 1) or vaccinia virus (strain CVI-79) (19) at a virus-to-cell ratio of 10. Uninfected

monolayers served as controls. Approximately 20 h after infection, the medium was removed and the cells were lysed by freezing and thawing three times. The lysate from approximately 2×10^8 infected or uninfected cells was resuspended in 20 ml of phosphate-buffered saline (PBS) containing calcium and magnesium, sonically treated (15 s), and centrifuged at 1,400 $\times$ *g* for 15 min. Supernatant fluids served as the source of viral or control antigens. Viral titers were determined by plaque assay on PRK cells (14). Semipurified HSV was prepared by differential and rate zonal centrifugation. Stock virus was clarified and then centrifuged in an SW30 rotor at 78,000 $\times$ *g* for 120 min. The pellet was resuspended in PBS and centrifuged in an SW41 rotor at 100,000 $\times$ *g* for 120 min through a 10 to 40% (wt/wt) linear sucrose gradient onto a cesium chloride cushion (1.8 g/ml). The appropriate fractions were pooled, dialyzed, and diluted 1:5 in PBS before use.

Antisera. Hyperimmune sera to HSV and vaccinia virus were prepared in rabbits, and the 50% plaque neutralization titers were approximately 1:2,000 (3). Normal rabbit serum was obtained from unimmunized rabbits. Immune serum globulin obtained from Cutter Laboratories, Berkeley, Calif., served as the source of human antibody against HSV. Unless indicated otherwise, rabbit anti-HSV serum was used in all experiments. Antiserum to rabbit immunoglobulin G (IgG) was prepared by hyperimmunizing goats with the purified IgG in Freund complete adjuvant. The IgG fractions of the various sera were prepared by ammonium sulfate precipitation and Sephadex G-200 chromatography (14). Rheumatoid factor (RF) was prepared from human serum by the method of Schrohenloher et al. (27), and the titer, as measured by agglutination of Latex particles coated with human globulins, was 1,280. Immunoblobulins were labeled with ^{125}I by the chloramine-T method (18, 23).

Direct micro-RIA. Twenty-five μliters of viral or control antigens, diluted 1:9 in PBS, were placed in wells of polyvinyl microtiter plates (Cooke Engineering Co., Alexandria, Va.). Each 3.5- by 5-inch plate contained 96 U-shaped wells. The antigens were allowed to dry at 37 C and then were fixed with 0.1 ml of absolute methanol for 5 min at room temperature. Twenty-five μliters of the appropriate dilutions of ^{125}I-labeled antiviral IgG was then added to the wells. Unless stated otherwise, all antibody dilutions in the direct and indirect RIA were made in growth medium, and all reactions were carried out for 1 h at 37 C. After incubation, the wells were washed 10 times with tap water. Each well then was separated from the microtiter plate with scissors, placed in a tube, and counted for radioactivity in a Packard Auto-Gamma spectrometer.

Inhibition assay. To microtiter plates containing viral and control antigens, 25 μliters of serial twofold dilutions of unlabeled anti-HSV serum was added. After 1 h at 37 C the serum was removed and ^{125}I-labeled anti-HSV IgG was added. The microtiter plates were placed at 37 C for 1 h and then washed 10 times, the individual wells were counted for radioactivity, and the percent of inhibition of binding of ^{125}I-labeled anti-HSV IgG was determined. The titer of the unlabeled serum was expressed as the reciprocal of the highest serum dilution that inhibited the binding of ^{125}I-labeled anti-HSV IgG by 50%.

Indirect micro-RIA. To microtiter plates containing viral or control antigens, 25 μliters of serial twofold dilutions of unlabeled antiviral serum was added. After 1 h at 37 C, the serum was removed and the plates were washed 10 times. Twenty-five μliters of ^{125}I-labeled goat anti-rabbit IgG or ^{125}I-labeled RF diluted 1:80 in growth medium was then added. After 1 h at 37 C, the plates were washed 10 times, and the individual wells were counted for radioactivity (Fig. 1). The binding ratios were determined by

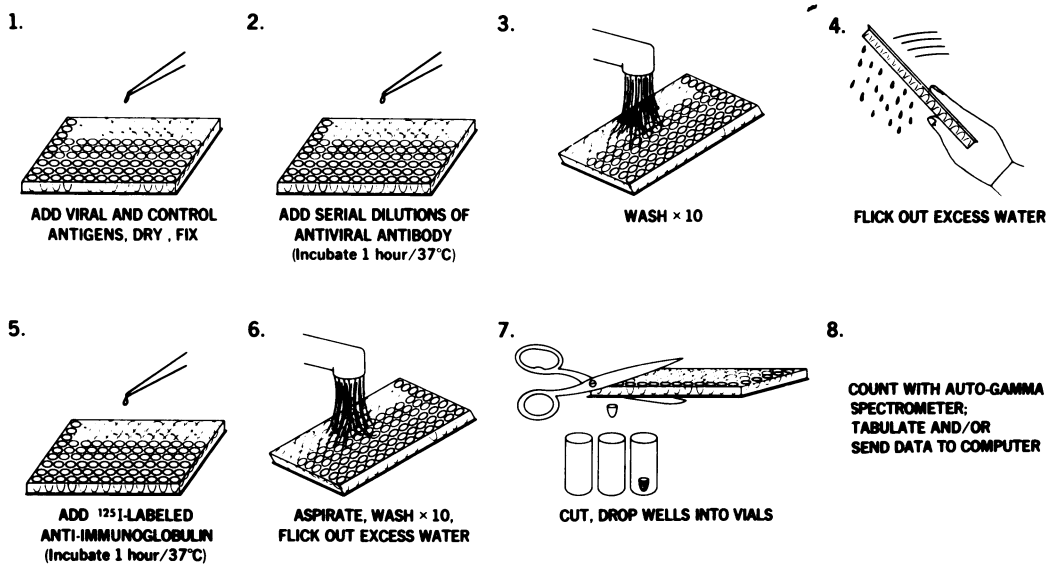

FIG. 1. *Schematic diagram of solid-phase indirect microradioimmunoassay.*

dividing the average counts per minute bound to wells containing viral antigens by the counts per minute bound to wells containing control antigens. The reciprocal of the highest serum dilution that resulted in a binding ratio equal to or greater than 1.5 was taken as the end point of the antiviral activity (26). All data are expressed as the average of duplicate reactions.

RESULTS

Detection of antiviral antibody by direct technique. The ability of ^{125}I-labeled antiviral IgG to bind specifically to viral antigens adherent to the surface of polyvinyl wells is shown in Table 1. Nearly 10 times as much ^{125}I-labeled anti-HSV IgG bound to wells containing HSV antigens than to wells containing vaccinia or control antigens. Similarly, more ^{125}I-labeled anti-vaccinia IgG bound to vaccinia than to HSV or control antigens.

Detection of antiviral antibody by inhibition of direct technique. The ability of unlabeled antiviral antibody to block the binding of ^{125}I-labeled anti-HSV IgG is illustrated in Fig. 2. Serial twofold dilutions of unlabeled anti-HSV serum were added to wells containing HSV or control antigens and then ^{125}I-labeled anti-HSV IgG was added. It can be seen that a 1:10 dilution of unlabeled anti-HSV serum inhibited the binding of labeled anti-HSV IgG by nearly 90%. Fifty percent inhibition was produced by a 1:40 dilution of unlabeled antiviral serum.

Detection of antiviral antibody by indirect technique. A comparison of the indirect micro-RIA (Table 2) with the direct inhibition technique (Fig. 2) shows that the former is far more sensitive. Whereas the titer of anti-HSV antibody by the direct inhibition technique was 40, the titer of the same serum by the indirect technique was 16,000. The data in Table 2 also show that, when ^{125}I-labeled RF was substituted for ^{125}I-labeled anti-IgG, specific binding did

TABLE 1. *Specificity of binding of ^{125}I-labeled antiviral antibody*[a]

Antigens	^{125}I-labeled anti-HSV IgG (counts/min)	^{125}I-labeled anti-vaccinia IgG (counts/min)
HSV	3,720	1,189
Vaccinia	367	2,962
Control	378	945

[a] Wells containing HSV, vaccinia, or control antigens were incubated for 1 h at 37 C with ^{125}I-labeled antiviral IgG (diluted 1:10 in growth medium). The wells then were washed and counted for radioactivity.

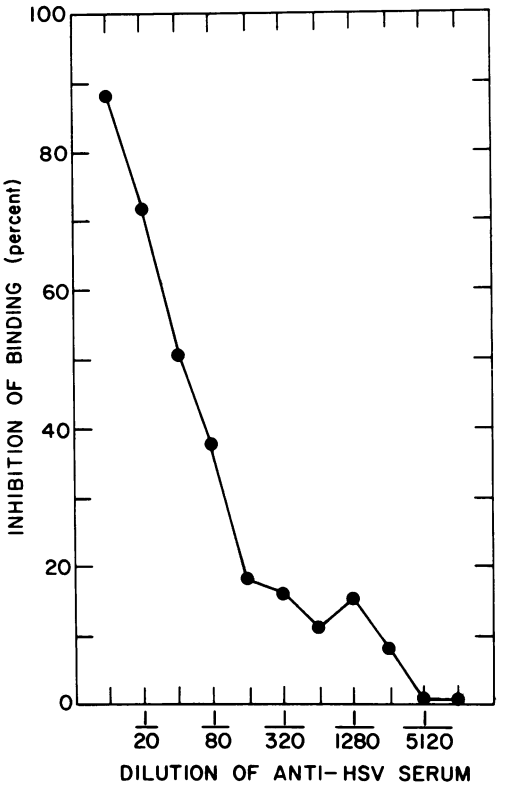

FIG. 2. *Inhibition of binding of ^{125}I-labeled anti-HSV IgG by unlabeled anti-HSV serum. Serial twofold dilutions of unlabeled anti-HSV serum were added to wells containing HSV or control antigens. After 1 h at 37 C the serum was removed, and ^{125}I-labeled anti-HSV IgG (diluted 1:8 in growth medium) was added. At the end of 1 h the wells were washed, and the inhibition of binding produced by the unlabeled serum was determined.*

TABLE 2. *Titer of antiviral antibody as measured by the binding of ^{125}I-labeled goat anti-rabbit IgG or ^{125}I-labeled rheumatoid factor (RF)*[a]

Viral antigens	Antiviral serum	^{125}I-labeled immunoglobulin	Titer of antiviral serum[b]
HSV	Anti-HSV (rabbit)	Anti-IgG	16,000
HSV	Anti-HSV (rabbit)	RF	256
HSV	Anti-HSV (human)	RF	512
Vaccinia	Anti-vaccinia (rabbit)	Anti-IgG	4,000
Vaccinia	Anti-vaccinia (rabbit)	RF	256

[a] Microtiter wells containing HSV, vaccinia, or control antigens were incubated for 1 h with serial twofold dilutions of each of the above antiviral serums, washed, and incubated for 1 additional h with ^{125}I-labeled anti-IgG or ^{125}I-labeled RF. The wells then were washed and counted for radioactivity.

[b] Titer represents the reciprocal of the highest serum dilution that resulted in a binding ratio equal to or greater than 1.5.

occur, but the detection of antiviral antibody by RF was considerably less sensitive. RF also bound to immune complexes composed of HSV and anti-HSV antibody of human origin.

Factors influencing specific binding of radiolabeled immunoglobulins. During the course of these experiments it became apparent that a number of factors could influence the sensitivity of both the direct and indirect RIA. Nonspecific attachment of ^{125}I-labeled immunoglobulins to polyvinyl wells resulted in a high background of radioactivity and a marked reduction in the apparent binding ratio. This could be largely prevented, however, by adding protein to the appropriate diluent. Wells containing semipurified HSV antigens were treated for 1 h at 37 C with PBS, 1% bovine serum albumin (BSA), or 1% calf serum and then incubated with ^{125}I-labeled anti-IgG diluted in PBS. Table 3 shows that wells which had been pretreated with 1% protein bound approximately one-half the amount of ^{125}I-labeled anti-IgG as wells pretreated with PBS. However, if the ^{125}I-labeled anti-IgG was diluted in 1% BSA or 1% calf serum instead of PBS, nonspecific attachment was reduced by up to 95%.

The effect of nonspecific attachment of ^{125}I-labeled anti-IgG on the binding ratio is demonstrated in Table 4. It can be seen that the binding ratio increased from 1.4 when PBS was used as the diluent to 7.5 when 10% growth medium was used as the diluent. The increase in the binding ratio was associated with a corresponding decrease in the nonspecific attachment of ^{125}I-labeled anti-IgG.

In contrast to the increase in the binding ratio which occurred when increasing concentrations of serum were used in the diluent for ^{125}I-labeled anti-IgG, a decrease in the binding ratio occurred when increasing concentrations of serum were used in the diluent for viral and control antigens during the initial fixation of these antigens to polyvinyl wells. Antigens were diluted in PBS containing various concentrations of BSA and then fixed to microtiter wells in the usual way. The binding of antiviral antibody was measured by indirect RIA. The data in Table 5 show that high concentrations of BSA markedly decreased the subsequent binding of ^{125}I-labeled anti-IgG and reduced the binding ratio from 11.3 to 1.9.

TABLE 3. *Nonspecific attachment of ^{125}I-labeled anti-IgG to microtiter wells*[a]

Initial treatment of microtiter wells	Diluent for ^{125}I-labeled anti-IgG (counts/min)		
	PBS	1% BSA	1% Calf serum
PBS	9,430	423	586
1% BSA	4,503	509	470
1% Calf serum	5,979	295	393

[a] Wells containing semipurified HSV antigens were incubated for 1 h at 37 C with PBS, 1% BSA, or 1% calf serum, washed, and then incubated for 1 additional h with ^{125}I-labeled anti-IgG diluted in PBS, 1% BSA, or 1% calf serum. The plates were washed 10 times, and the amount of ^{125}I-labeled anti-IgG that bound was determined.

TABLE 4. *Effect on nonspecific attachment of ^{125}I-labeled anti-IgG on the binding ratio*[a]

Diluent for ^{125}I-labeled anti-IgG	Anti-HSV antibody (counts/min)	Normal serum (counts/min)	Binding ratio
PBS	3,920	2,714	1.4
0.25% BSA	1,047	472	2.2
2.0% BSA	1,032	402	2.6
1.0% BSA	1,115	356	3.1
10% Calf serum	777	126	6.1
10% Growth medium	668	89	7.5

[a] Anti-HSV antibody or normal rabbit serum, diluted 1:100 in 1% BSA, was added to wells containing semipurified HSV antigens. At the end of 1 h the wells were washed and incubated for 1 additional h with ^{125}I-labeled anti-IgG which had been diluted 1:80 in each of the above diluents. The wells then were washed and counted for radioactivity.

TABLE 5. *Effect of reagents used to dilute viral and control antigens on the binding of antiviral antibody*[a]

Reagents used to dilute antigens	Viral antigens (counts/min)	Control antigens (counts/min)	Binding ratio
PBS	1,774	156	11.3
0.03% BSA	1,464	146	10.0
0.06% BSA	1,301	122	10.6
0.125% BSA	939	96	9.7
0.25% BSA	612	90	6.8
0.5% BSA	294	86	3.4
1.0% BSA	128	70	1.8
10.0% Calf serum	125	64	2.0
Growth medium	110	58	1.9

[a] Vaccinia and control antigens were diluted 1:9 with each of the above reagents and then fixed to microtiter wells in the usual way. Anti-vaccinia antibody (diluted 1:200 in growth medium) was added. At the end of 1 h the wells were washed, incubated for 1 additional h with ^{125}I-labeled anti-IgG (diluted 1:80 in growth medium), washed again, and counted for radioactivity.

Measurement of viral antigens by indirect technique. Preparations containing viral or control antigens were diluted serially in PBS, fixed to microtiter wells, and quantitated by the indirect RIA. Fig. 3 shows that, at the lower dilutions of antigen, considerably more radioactivity bound to wells containing viral than control antigens. Marked differences still could be detected at a 1:128 dilution of the viral antigens.

DISCUSSION

Previously we showed that ^{125}I-labeled antiviral antibody could be used to detect viral antigens on the surface of infected cells (15; Hayashi, Lodmell, Rosenthal, and Notkins, J. Immunol., in press; Rosenthal, Hayashi, and Notkins, J. Gen. Virol., in press) and that viral antigens extracted from infected cells adhered to the surface of polyvinyl wells (26). The present experiments show that ^{125}I-labeled antiviral antibody will bind to the adherent antigens (Fig. 4A). Although the amount of ^{125}I-labeled antibody which binds to the viral antigens could be used to measure the titer of the antiviral antibody, this technique is not practical for screening a large number of samples because each serum would have to be individually labeled. The inhibition of binding of ^{125}I-labeled antiviral antibody by unlabeled antiviral antibody (Fig. 4B) avoids this difficulty, but, at least under the conditions employed here, the sensitivity is low. In contrast, the indirect technique is very sensitive and has the advantage of using one labeled reagent for detecting antibody to a variety of viruses. Presumably, the greater sensitivity of the indirect technique is due to the amplification resulting from the attachment of more than one molecule of ^{125}I-labeled anti-immunoglobulin to each molecule of unlabeled antiviral antibody (Fig. 4C).

A number of factors can affect the sensitivity of these assays. First, nonspecific binding of ^{125}I-labeled anti-immunoglobulin to polyvinyl wells can obscure specific differences in binding. Nonspecific binding, however, can be reduced by adding BSA to the labeled anti-immunoglobulin. The BSA apparently competes with the labeled immunoglobulin for nonspecific binding sites on the surface of the polyvinyl wells. In our experiments, the addition of BSA to the ^{125}I-labeled immunoglobulin was markedly more effective in preventing nonspecific binding than pretreatment of the wells with BSA or calf serum (Tables 3 and 4). This might be due to the loss of protein "coat" from the surface of the wells as a result of repeated washings.

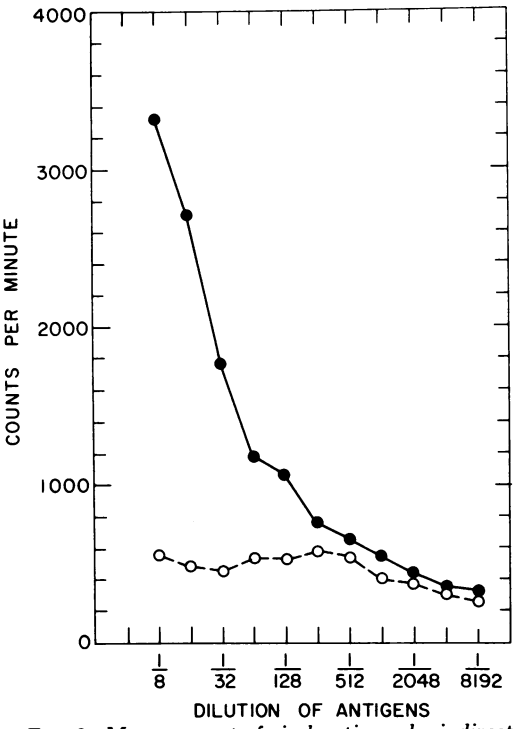

FIG. 3. *Measurement of viral antigens by indirect RIA. Serial twofold dilutions of HSV antigens (●) or control antigens (○) in PBS were dried and fixed to microtiter wells. Anti-HSV serum (diluted 1:500 in growth medium) then was added. At the end of 1 h the wells were washed and incubated for an additional h with ^{125}I-labeled anti-IgG (diluted 1:80 in growth medium). The wells were washed and counted for radioactivity.*

The conditions under which the viral antigens are allowed to adhere to the surface of the polyvinyl wells also are important. The highest binding ratio was obtained when the antigens were diluted in medium free of protein. When the antigens were diluted in medium containing protein, the binding ratio was greatly reduced. Whether the protein competes with the viral antigens for sites on the surface of the wells or whether the protein covers the viral antigens and makes them inaccessible to antibody is not certain. There also is some evidence that, if the viral antigens are fixed in the presence of a high concentration of protein, the antigens are more readily washed off the plate (unpublished observations). Thus, the highest binding ratio is obtained when the diluent for the antigen contains little protein, while the diluent for the labeled anti-immunoglobulin contains high protein.

RF, which is capable of binding to immune

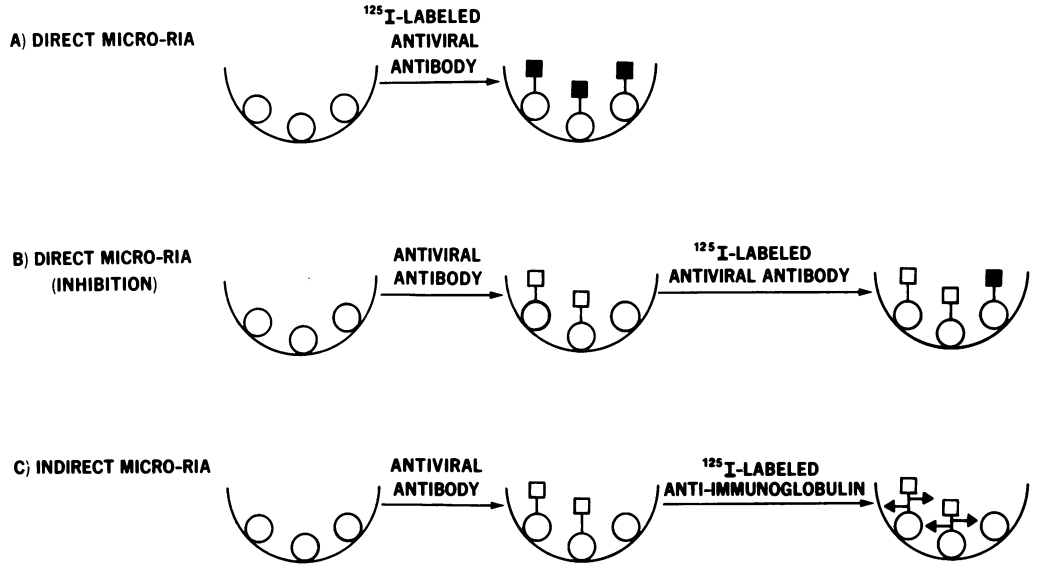

FIG. 4. *Comparison of direct and indirect solid-phase microradioimmunoassay. Direct micro-RIA: wells containing viral antigens (open circles) are incubated with ^{125}I-labeled antiviral antibody (closed boxes). Inhibition of direct micro-RIA: wells containing viral antigens are incubated with serial dilutions of unlabeled antiviral antibody (open boxes) and then ^{125}I-labeled antiviral antibody. Indirect micro-RIA: wells containing viral antigens are incubated with serial dilutions of unlabeled antiviral antibody and then ^{125}I-labeled anti-immunoglobulin (closed triangles).*

complexes on the surface of virus-infected cells (Hayashi, Lodmell, Rosenthal, and Notkins, J. Immunol., in press) and to infectious virus-antibody complexes (2), also is capable of reacting with immune complexes in the solid-phase micro-RIA. The titer of antiviral antibody as measured by this technique was considerably lower than that obtained with ^{125}I-labeled anti-immunoglobulin. It should be emphasized, however, that the anti-immunoglobulin was prepared by hyperimmunization and presumably has a greater affinity for immunoglobulins than RF has for immune complexes. In theory, at least, one possible advantage of RF over anti-immunoglulin is that the former reacts with immune complexes, but not native immunoglobulin. Thus, ^{125}I-labeled RF might prove useful in the detection of immune complexes. The ability of RF to bind to immune complexes, however, points to potential problems that might arise with the indirect micro-RIA technique. Since RF is found in about 1% of the population (16), serum containing antiviral antibody also might contain RF. Thus, RF might bind to antiviral antibody once the latter forms a complex with viral antigens. This might interfere with the subsequent attachment of ^{125}I-labeled anti-immunoglobulin. Whether this will prove to be a real problem or whether the concentration of RF in most serums is so low that it will be diluted out before the end point of the antiviral antibody is reached remains to be established.

The indirect technique also might be used to determine the class of the antiviral immunoglobulin bound to the viral antigens by employing specific anti-immunoglobulins (e.g., anti-IgG, anti-IgM, anti-IgA) (8, 11). In fact, to avoid false negatives in detecting and titrating antiviral antibody, the routine preparation of ^{125}I-labeled anti-immunoglobulin probably should contain antibodies against the several different immunoglobulin classes.

In conclusion, solid-phase micro-RIA procedures seem to have a number of advantages over many of the commonly used serological techniques and with modifications and refinements should find broad application in viral serology. In each case, optimal conditions for the adherence of viral antigens to the microtiter wells must be carefully worked out, and nonspecific binding of radiolabeled immunoglobulin must be prevented. Greater specificity and sensitivity will be achieved, undoubtedly, by employing highly purified viral antigens and highly specific radiolabeled immunoglobulins.

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