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A Simple and Sensitive Solid-Phase Radioimmunoassay for the Assay of Human TSH Antibody

Use in Screening Hybridoma Cell Lines

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Abstract

A simple and sensitive radioimmunoassay procedure is described for the screening and detection of specific antibodies in hybridoma cell lines. The specific procedure was developed to screen for antibodies against human thyrotropin (hTSH), but the procedure is applicable to screening for any desired antibodies. The immunoglobulin G (IgG) fraction of goat anti-mouse IgG is used to coat wells of microtiter plates. Anti-hTSH antibodies are measured by incubating antiserum dilutions in the coated wells and detecting the bound IgG with radioiodinated hTSH. Unlabeled hTSH may also be detected by its ability to inhibit binding of ^{125}I-hTSH to the coated wells. This assay technique meets the demands of simplicity, sensitivity, reproducibility, and rapidity as a screening assay of hybridoma cell lines capable of secreting anti h-TSH.

Index Entries: Human TSH; solid-phase radioimmunoassay; anti-mouse immunoglobulin G; microtiter plate; radioiodinated hTSH; hybridoma cell lines; monoclonal anti-hTSH; radioimmunoassay, for human TSH antibody; thyrotropin, screen for antibodies to human; antibody, RIA for human TSH.

Introduction

The glycoprotein hormones, TSH,† LH, FSH, and CG, consist of immunologically and biologically unique β-subunits noncovalently associated with immunologically identical α-subunits (*1*). Antisera prepared against these hor-

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†Abbreviations used: TSH = thyroid stimulating hormone; LH = luteinizing hormone; FSH = follicle stimulating hormone; CG = chorionic gonadotropin.

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mones often show variable degrees of affinity with the common α-subunits, and may therefore suffer from a lack of specificity. Rarely, antisera produced against these hormones show high degrees of specificity, but large numbers of animals must be immunized and screened (*2*). The technique of somatic cell fusion has made it possible to prepare cell clones producing highly specific antibodies against these glycoprotein hormones (*3, 4*) or any other proteins. However, this technique has generated a need for new techniques suitable for rapid and sensitive screening of established hybridoma cell lines to permit the selection of those of desired specificity and affinity. Of these, the enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with antigen is by now a well-established technique for screening monoclonal antibodies (*5–7*). This solid-phase assay, however, does not appear to be useful for screening for antibodies against protein hormones, because of a great demand for the expensive protein hormones used to coat the plates. We report here a simple, accurate, and sensitive solid-phase radioimmunoassay (RIA) using microplates coated with anti-mouse immunoglobulin G (IgG), which was developed to permit determination of human TSH (hTSH) antibody in hybridoma cultures and mouse antisera. The technique is usable for rapid screening for any specific antibody, if a purified antigen is available in small amounts.

Materials and Methods

Solid-Phase RIA

The IgG fraction of goat anti-mouse IgG (Cappel Laboratories, Inc., PA) was adsorbed to the walls of 96-well microplates (Dynatech Laboratories, Inc., VA) by adding 0.1 mL/well of an antibody solution in 0.01*M* phosphate-buffered saline (PBS, pH 8.0) and incubating at 4°C for 20–24 h. After removing the antibody solution, 0.1 mL of PBS containing 1% bovine serum albumin (BSA) was added to each well, and the plates were covered with parafilm and stored at 4°C until used. Before using, PBS-BSA was removed and the plates were washed three times with distilled water. Samples including hybridoma cultures and mouse antisera were diluted as desired† with PBS-BSA; 0.05 mL was added to the wells, followed by the addition of a PBS-BSA solution (0.05 mL) containing about 15,000 cpm of ^{125}I-hTSH (specific activity, 360 μCi/μg). After incubation overnight at room temperature, the wells were washed five times with distilled water. Then the wells were cut and counted for radioactivity by a gamma counter (Packard Autogamma-800, Packard, IL).

Liquid-Phase RIA

For comparison, liquid-phase RIA was also performed in a test tube (borosilicate glass [12 × 75mm], American Scientific Products, IL) for the same samples. Culture medium or mouse serum diluted with PBS-BSA was reacted with about

†Generally in 1 to 3 serial decreasing dilutions.

10,000 cpm of ^{125}I-hTSH in a final volume of 1 mL containing 0.05*M* sodium phosphate, 0.25% BSA, and 0.1% bovine gamma-globulin, pH 7.5. The reaction mixture was precipitated after a 2-d incubation at 4°C with 1 mL of a 24% polyethylene glycol (PEG) 6000 solution (*8*). After centrifugation, the pellet was measured by a gamma counter.

Hybridoma Cultures

A saline solution of hTSH (NIAMDD, hTSH-RP-1) was emulsified in an equal volume of complete Freund's adjuvant, and 10 μg hTSH was injected at 20–30 multiple intradermal sites along the dorsal surface of mice. The mice were injected intravenously with 10 μg of hTSH in 0.1 mL of PBS 3 d before the removal of the spleen. The fusion procedures were performed as described by Galfre et al. (*9*).

Results

Anti-Mouse IgG Concentration for Coating of Microplates

Microplates were sensitized with various concentrations of anti-mouse IgG and used in the solid-phase RIA for determining hTSH antibody titers in hybridoma cultures and mouse sera (Fig. 1). Antibody titers were expressed as % bound radioactivity of ^{125}I-hTSH added to each well. Nonspecific binding ranged from 0.05 to 0.28% with mean ± SD of 0.12 ± 0.09% for ten determinations.

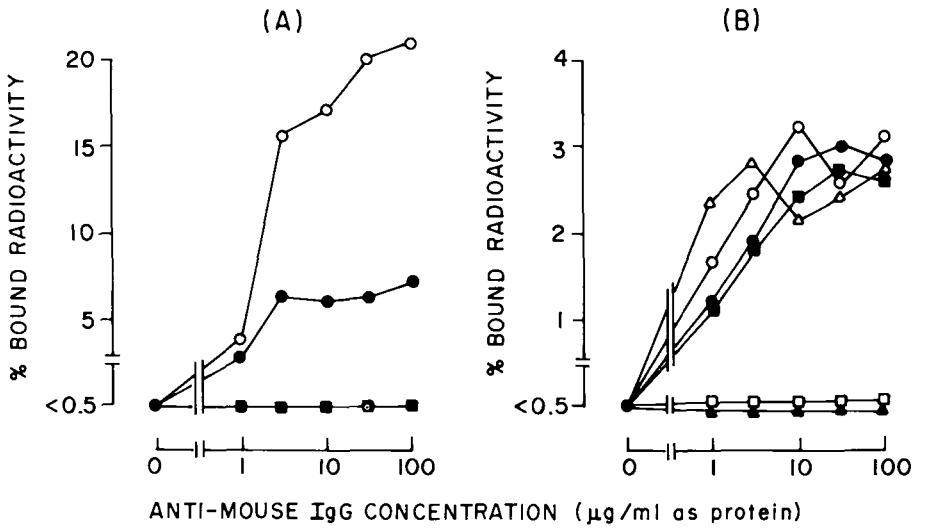

Fig. 1. Solid-phase RIA using ^{125}I-hTSH and microplates coated with various concentrations of goat anti-mouse IgG. (A) Mouse sera from in vivo immunizations: (○, positive serum at a final dilution of 1:20,000; ●, positive serum at a final dilution 1:200,000; ■, negative serum at a final dilution of 1:20,000). (B) Culture media of fused cells (■, ●, ○, △, positive media at a final dilution of 1:15; □, ▲, negative media at a final dilution of 1:5)

Anti-hTSH antibody present in positive serum bound, in microplates coated with 3–100 μg/mL of an anti-mouse IgG solution, 16–22% of added labeled hTSH at a final dilution of 1:20,000 and about 7% of that even at a final dilution of 1:200,000. In contrast, sera containing no antibody bound less than 0.5% (0.12–0.40%) at a final dilution of 1:20,000 in the use of any plate. Four culture media from growing clones, which are positive for anti-hTSH in the liquid-phase RIA, showed 1.8–3.2% binding of ^{125}I-hTSH at a final dilution of 1:5 when coating was carried out at more than 3 μg/mL of an anti-mouse IgG solution, while negative culture media showed negligible binding activity of less than 0.5% (0–0.36%). Microplates coated with more than 3 μg/mL of an antibody solution gave similar results for screening the culture media, although 1 μg/mL concentration was insufficient to give sensitive plates. These results show that 3 μg/mL of an anti-mouse IgG solution was appropriate to coat microplates, and this concentration was adopted for further coating.

Effect of pH of an Anti-Mouse IgG Solution for Coating of Microplates

Anti-mouse IgG was diluted with PBS of pH 8.0 and 0.1*M* carbonate buffers of pH 9.0 and 9.7. These three different pH antibody solutions were used for coating microplates. After washing, these plates were applied to the solid-phase RIA in order to titrate mouse anti-hTSH serum according to the procedures described in Methods and Materials. As shown in Fig. 2, higher titers were obtained when coat-

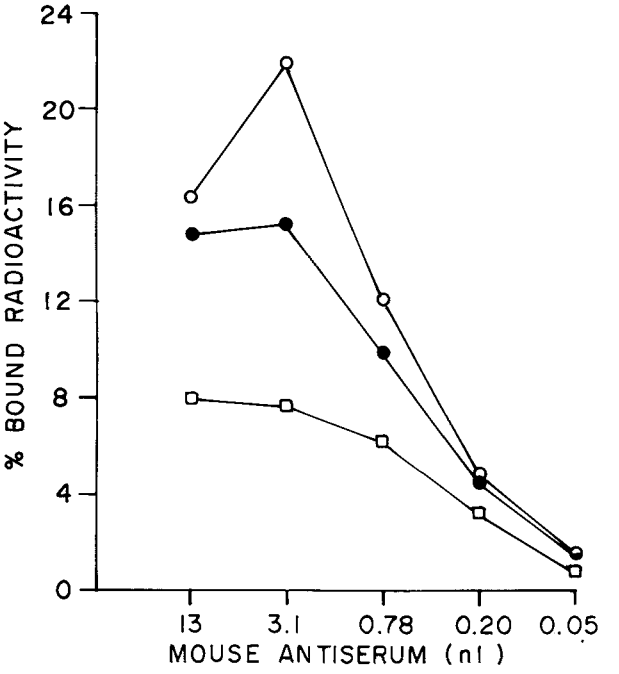

Fig. 2. Antiserum dilution curves in the solid-phase RIA using microplates coated with anti-mouse IgG diluted in 0.01*M* phosphate buffered saline of pH 8.0 (○), and 0.1*M* carbonate buffers of pH 9.0 (●) and 9.7 (□).

ing was performed at pH 8.0 rather than at pH 9.0 or 9.7. More antibody was adsorbed to the walls of plates in PBS at pH 8.0 than in carbonate buffers of pH 9.0 and 9.7.

Antibody Dilution Curves

Anti-hTSH antisera obtained by tail bleeding of the intact mice and antibody from positive culture medium of fused cells were serially diluted with PBS-BSA and assayed in both RIAs, using the solid-phase method with anti-mouse IgG-coated plates and the usual liquid-phase method, using a PEG solution for separation of antibody bound from free hormone. All dilution curves obtained using both types of RIAs were parallel (Fig. 3). Antibody present in 0.16 nL of the antiserum (final dilution, 1: 600,000) was detected in the solid-phase RIA as well as in the liquid-phase RIA. However, the bound radioactivity (1.3%) was about six times less than that (8.3%) obtained using the liquid-phase method. The nonspecific binding in the former method (0.12 ± 0.09%), however, was much less than that in the latter method (4.9 ± 1.3%), and therefore the solid-phase RIA was found to be as sensitive as the liquid-phase RIA.

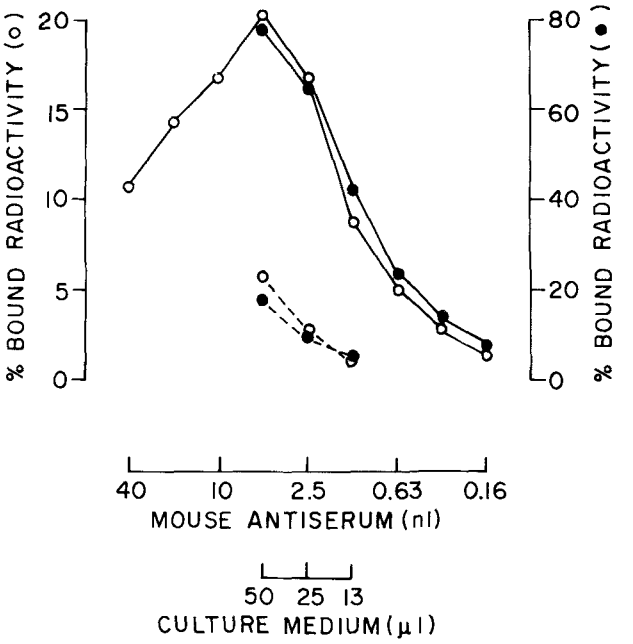

Fig. 3. Antibody dilution curves. An antiserum from a mouse immunized in vivo and bled via tail vein was diluted and tested by the microwell method (○) and the usual liquid immunoassay (●) (top two solid lines). Antibodies from cultured fused cells were tested by the microwell method (○) and the usual liquid immunoassay (●) (bottom two dashed lines). The left ordinate shows percent radioiodinated TSH bound to antibodies from hybridomas; the right ordinate shows percent radioiodinated TSH bound to antiserum obtained from the whole mouse.

Bound radioactivity remarkably decreased when serum volume was increased from 5 to 40 nL in the solid-phase RIA. The most likely reason for this was that at high antibody concentrations, anti-hTSH antibody was in excess of the anti-mouse IgG bound to the wells. The excess anti-hTSH would bind to radioiodinated hTSH, but would not adhere to the plate.

Specificity of the Solid-Phase RIA

Two series of studies were performed to check the specificity of the present solid-phase RIA. In the first, various amounts of the culture media containing mouse IgG, which proved unreactive toward hTSH, were added both to the assay diluent (PBS-BSA) and to a reference anti-hTSH solution dissolved in PBS-BSA, and then assayed for antibody titers. As shown in Fig. 4, nonspecific IgG in the two different culture media had no effect on the nonspecific binding (0.20–0.41%), whereas the binding of specific IgG was consistently decreased by increasing amounts of nonspecific IgG. These results showed that there was no false positive reaction caused by the presence of nonspecific IgG in some hybrid supernatants. However, competition between specific and nonspecific antibodies was present and decreased the bound radioactivity. However, even in the presence of 1500 ng/

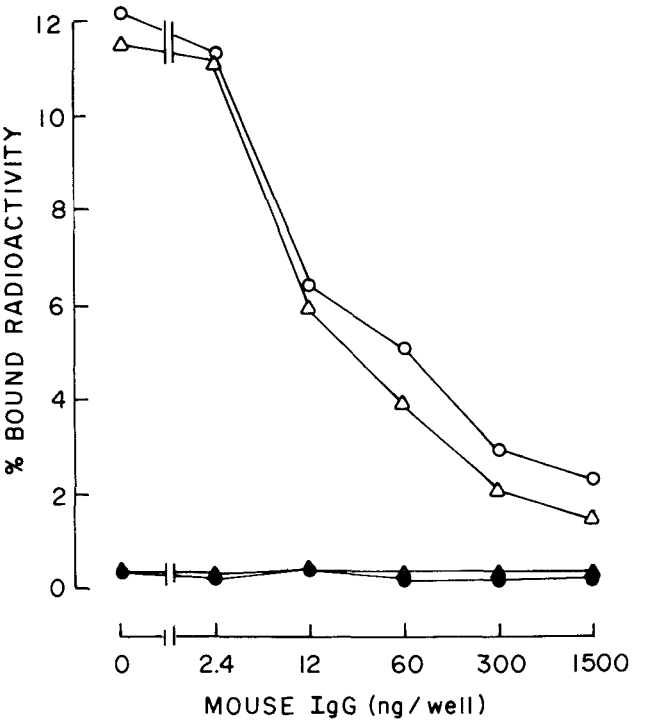

Fig. 4. Effect on solid-phase RIA of increasing amounts of mouse IgG (unreactive toward hTSH) in two different culture media [D 1 (△ and ▲) and T 6 (○ and ●)] added to anti-hTSH serum diluted with the assay diluent (△ and ○) and to the assay diluent alone (▲ and ●).

well (30 μg/mL) nonspecific IgG, the data showed the media to be positive for specific antibodies.

In the second series, a dilution of anti-hTSH antibody that bound approximately 12% of added ^{125}I-hTSH in the anti-mouse IgG-coated well was determined. Varying doses of unlabeled hTSH, ^{125}I-hTSH, and this dilution of anti-hTSH were then added to solid-phase anti-mouse IgG-coated wells and incubated at room temperature overnight. The wells were washed and counted for their bound radioactivity. Unlabeled hTSH significantly inhibited binding of ^{125}I-hTSH to anti-hTSH bound to the wells at amounts between 20 and 5000 pg/well (Fig. 5). These data suggest that the binding of ^{125}I-hTSH to the wells was caused by specific immunoreaction between labeled hTSH and anti-hTSH bound to the wells, and that the present solid-phase RIA is also applicable to measure hTSH antigen.

Coefficients of Variation in the Solid-Phase RIA

In order to investigate the reproducibility and precision of the RIA using microplates coated with anti-mosue IgG, the coefficients of variation were meas-

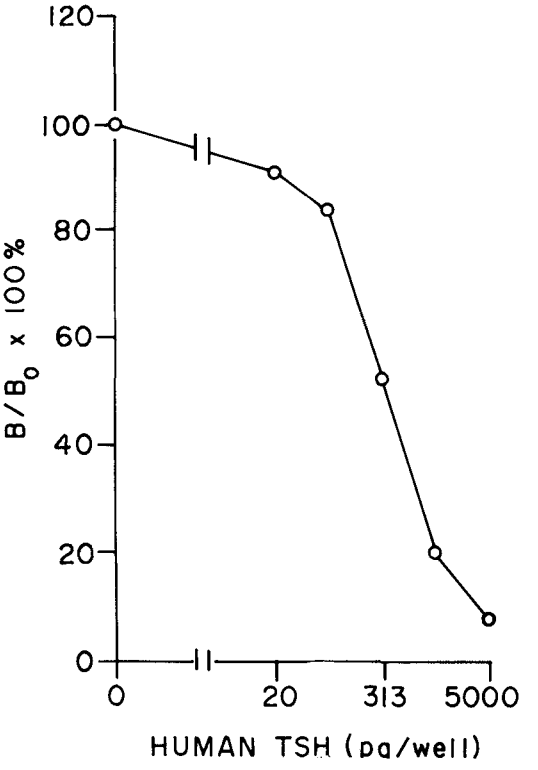

Fig. 5. Dose–response curve of unlabeled hTSH in the solid-phase RIA using ^{125}I-hTSH and anti-mouse IgG-coated microplates. Anti-hTSH serum was diluted until it could bind approximately 12% of the added radioactivity, and was added to the wells. The dose–response curve is plotted as B (percent of specific binding of the labeled hTSH in the presence of unlabeled hTSH) divided by B_0 (percent of specific binding of the labeled hTSH in the absence of unlabeled hTSH).

TABLE 1
Coefficients of Variation (CV) in the Solid-Phase RIA Using Anti-Mouse IgG-Coated Microplates

	Intra-assay (n = 8)[b]		Inter-assay (n = 5)[b]	
Sample[a]	% Bound radioactivity	CV, %	% Bound radioactivity	CV, %
1	1.84 ± 0.29	15.8	—	—
2	2.45 ± 0.31	12.7	—	—
3	2.93 ± 0.40	13.7	2.75 ± 0.47	17.1
4	3.11 ± 0.32	10.3	—	—
5	3.34 ± 0.27	8.1	3.28 ± 0.31	9.5
6	3.57 ± 0.31	8.7	3.44 ± 0.72	20.9
7	6.37 ± 0.50	7.8	—	—
8	8.38 ± 0.57	6.8	8.71 ± 0.73	8.4
9	8.65 ± 0.38	4.4	—	—
10	10.10 ± 0.32	3.2	10.03 ± 0.42	4.2
		Mean = 9.2%		Mean = 12.0%

[a]Culture media from growing clones were assayed.
[b]Samples were determined 8 times in intra-assay and 5 times in inter-assay.

ured at ten different antibody levels in culture media of fused cells (Table 1). They were 3.2–15.9% with an average of 9.2% for eight determinations in the intra-assay, and 4.2–20.9% with an average of 12.0% for five determinations in the inter-assay. These results show that the immunoassay is reproducible and precise.

Comparison of Solid-Phase and Liquid-Phase RIAs

Anti-hTSH titers in 64 samples, including 41 culture media and 23 sera from immunized mice, were determined by both the solid-phase plate method and the usual liquid-phase method using a 24% PEG solution for the separation of bound and free. Culture media and antisera were diluted 1:2 and 1:40,000 with PBS-BSA, respectively, and 0.05 mL portions of these samples were assayed for anti-hTSH. There was a good correlation (r = 0.924) between the values determined by the two methods (Fig. 6). The regression line determined by the method of least-squares was $y = 0.243x + 0.37$. The values in the solid-phase method were approximately four times less than those in the liquid-phase method.

Discussion

We have developed a simple and sensitive RIA for screening anti-hTSH monoclonal antibody. Previously, ELISA has been employed as a screening assay of antibody-secreting hybridoma cell lines. Antigen-coated plates are used in the ELISA and 20 μg–1 mg of antigen is needed to prepare one plate. This technique was not adopted for determination of anti-hTSH because of a limited supply of

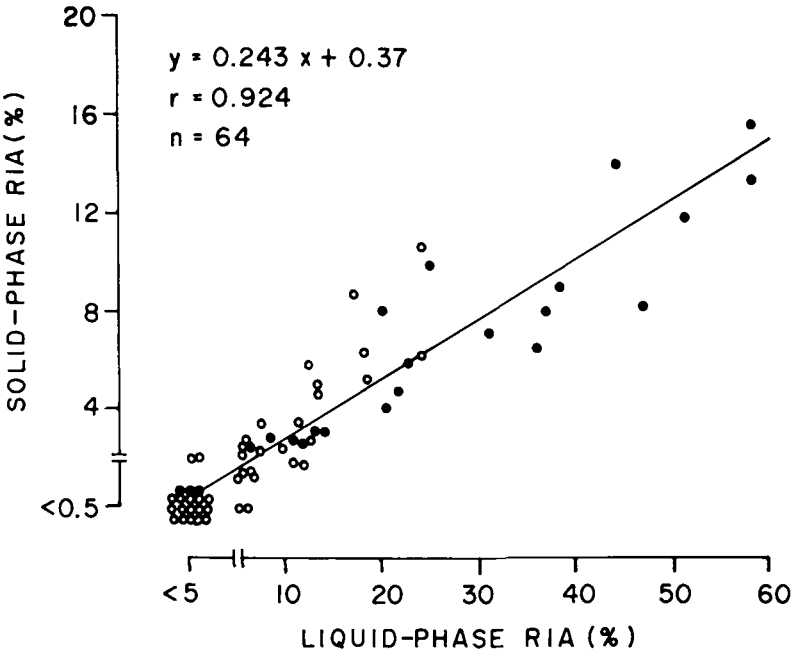

Fig. 6. Comparison of hTSH antibody titers obtained by solid-phase and liquid-phase RIAs using radioiodinated hTSH. In the solid-phase method, anti-mouse IgG-coated microplates were used, and in the liquid-phase method a 12% polyethylene glycol solution was used for separation of antibody bound from free ^{125}I-hTSH. Antibody titers were expressed as %bound radioactivity of added ^{125}I-hTSH. Sixty-four samples, including 41 culture media of fused cells (○) and 23 sera from immunized mice (●) were assayed in both methods. The regression line determined by the method of least-squares has a slope of 0.243 and an intercept of 0.37%. The correlation coefficient for these points is 0.924.

hTSH preparations. In the present solid-phase RIA, however, antimouse IgG-coated plates were used and only 5–10 ng of ^{125}I-hTSH was needed to assay 100 samples in duplicate. Moreover, the present method is easier to perform than ELISA, because sample solutions and labeled hTSH can be mixed simultaneously in wells coated with anti-mouse IgG, and the bound radioactivity can be counted immediately after incubating and washing.

ELISA needs two-step incubations, where samples containing antibody are added to antigen-coated wells and non-antibody IgG are washed away after incubation, and enzyme-labeled anti-mouse IgG is added to the wells.

This solid-phase RIA was compared to the liquid-phase RIA. Maximal binding activity of ^{125}I-hTSH was approximately 22% in the solid-phase method, whereas it was 75–80% in the liquid-phase method. Bound radioactivities of various samples obtained in the solid-phase method were also about four times less than those in the liquid-phase method. This is explained by the fact that fewer protein binding sites are present on the walls of wells and the amount of anti-mouse IgG adsorbed to the walls is limited (Figs. 1, 6). For this reason, samples containing non-antibody IgG should be assayed after appropriate dilution, since the binding of antibody IgG is inhibited, as shown in Figs. 3 and 4. Boniolo et al. (*10*) also dem-

onstrated a competition between specific and nonspecific antibodies and the resulting false negatives, although they used polystyrene balls as solid phase. On the other hand, nonspecific binding of the solid-phase method was negligible and 40 times less than that of the liquid-phase method. Therefore, the present solid-phase method gave similar sensitive results, although bound radioactivities were four times less.

The present RIA is easily applicable to assay IgM or IgG antibody of species other than mice by using microplates coated with antibody against IgM or IgG of the respective species. Additionally, it gives simple, sensitive, rapid, and reproducible screening assays for monoclonal antibodies against any other proteins that are available in small amounts in highly purified form suitable for radioiodination or labeling by other means.

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