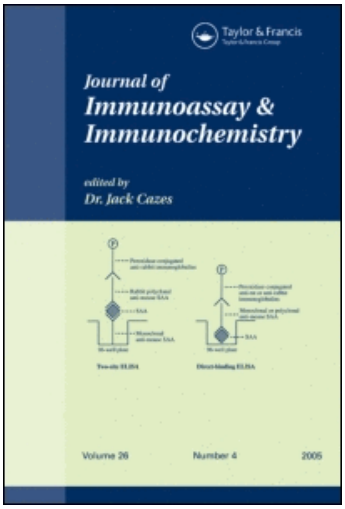

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IgG and IgM Antibodies to Rubella Quantitated by Enzyme Immunoassay

Roy B. Johnson Jr[a]; Fred C. Jensen[b]; Christopher R. Peter[c]; Robert M. Nakamura[a]
[a] Department of Pathology, Green Hospital of Scripps Clinic, La Jolla, California [b] Department of Immunopathology, Research Institute of Scripps Clinic, La Jolla, California [c] Department of Health Services, Laboratory, San Diego County, San Diego, California

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IgG AND IgM ANTIBODIES TO RUBELLA QUANTITATED BY ENZYME IMMUNOASSAY

Roy B. Johnson, Jr[1], Fred C. Jensen,[2],

Christopher R. Peter[3], and Robert M. Nakamura[1]

[1]Department of Pathology, Green Hospital of Scripps Clinic,

10666 North Torrey Pines Road, La Jolla, California 92037.

[2]Research Institute of Scripps Clinic, Department of Immunopathology,

10666 North Torrey Pines Road, La Jolla, California 92037.

[3]Laboratory, San Diego County Department of Health Services,

1600 Pacific Highway, San Diego, California 92101.

ABSTRACT

A solid-phase enzyme immunoassay for rubella antibodies (of IgG, H & L type, IgM-type) is described that requires assay at usually one dilution of serum. Results are reportable in milligrams IgG (or IgM) - equivalents per liter serum and in approximate hemagglutination inhibition (HAI) titers. The method uses purified rubella virus immobilized by a special process in excess, a 16 to 18 h first incubation at 20°C, and a glucose-oxidase labeled second antibody to obtain 97% agreement with HAI. IgM-class antibodies are assayed after a preliminary separation from IgG by ion exchange to obtain results equivalent to those obtained after sucrose gradient sedimentation.

INTRODUCTION

Use of solid-phase enzyme immunoassay (SP-EIA) for rubella antibody in serum requires special conditions to achieve simplicity, accuracy, and reliability. Earlier workers have contributed toward this end by demonstrating the need for purified antigen (1) and by initiating means to translate the absorbance (due to the colored product of enzyme action) into quantity of antibody (2,3). Although most reported methods distinguish moderately large differences in antibody concentration, the ability to distinguish small differences at all concentration levels and with simplicity has not previously been shown. Indeed, the great theoretical possibility of SP-EIA to simplify the assay and to specify the class of antibody reliably have not previously been realized.

We wish to report a series of conditions and techniques that allow assays of rubella antibody, including IgM-type specifically, to be performed at usually a single dilution with results reportable both in milligrams IgG (or IgM) - equivalents per liter-serum and in approximate hemagglutination inhibition (HAI) titer-equivalents. This simplified method shows a 97% agreement with the standard accepted assay for rubella antibody (HAI) as described by the Center for Disease Control, Atlanta, Georgia.(4)

MATERIALS AND METHODS

Sera: We used SP-EIA to test 294 serum samples previously assayed by HAI (4,5) at the San Diego County Health Department. The sera included 27 samples from infants, 136 from 47 paired sera of persons with possible acute/convalescent phases of rubella

infection and 131, mostly from young women, tested to determine the status of their immunity. Positive and negative control sera for use in Rubella HAI tests were obtained from Gibco Diagnostics, Grand Island, N.Y. 14072 and from Flow Laboratories, Inc., McLean, VA. 22102.

Virus antigen: Rubella, type RA 27/3, obtained from Stanley Notkin, Wistar Institute, Philadelphia, PA and the RV strain from Natalie Cremer, California State Department of Health, were propagated in roller cultures of BHK-21 cells or African Green Monkey kidney (Vero) cells infected at a multiplicity of 1 plaque-forming unit/cell. At 24 h after infection, the cultures were fed fresh nutrient (RPMI-fetal calf serum). The culture fluids were harvested 4 days after infection, passed through a 0.45 micron Millipore filter (Millipore Corp., Bedford, MA. 01730) and sedimented at 105000 g in a SW27 rotor onto a 50% sucrose cushion. The virus was next sedimented through a preformed sucrose density gradient (10 to 50%) at 155000 g for 18 h. Purified virus was collected, resuspended in phosphate-buffered saline (PBS), pH 7.2, and pelleted at 155000 g for 4 h. The preparation of virions resuspended in fresh PBS, was divided into glass ampules and stored at -90°C.

The purified virus was titered by immobilizing increasing dilutions in wells of a microtiter plate and using that dilution just exceeding the capacity to bind 50 µl of antibody from a 1:64 (or later 1:100) dilution of control serum having an HAI titer of 1:256 (Table 1). Control sera of lower HAI titers including $\leq$1:8

TABLE 1.

Titration of purified rubella virus antigen prepared in Vero cells. One hour incubations at room temperature; 30 minute reaction at 20°C with buffer substrate. Best dilution: 1:50.

Serum Sample	Stated HAI Titer	Dilutions of rubella virus					
		1:20	1:40	1:50	1:60	1:80	1:100
Control[a]	-	0.035[b]	0.029	0.032	0.028	0.031	0.029
Flow Neg	<1:8	0.117	0.107	0.111	0.103	0.127	0.109
Gibco Neg	<1:8	0.259	0.244	0.248	0.249	0.266	0.265
Gibco Lo	1:16-1:32	0.433	0.402	0.430	0.383	0.407	0.386
Flow Lo	1:16	0.209	0.192	0.200	0.170	0.163	0.171
Flow Hi	Not given	0.684	0.519	0.697	0.588	0.622	0.608
Gibco Hi	1:256	0.952	0.914	0.906	0.853	0.823	0.753

[a]Control contained only reaction between antigen and enzyme-coupled antihuman IgG.

[b]Absorbancies at 410 nm vs H_2O reference.

were also used in the antigen titration to test the antigens ability to cause appropriate distinguishing results.

Early assays were performed using virus grown in BHK-21 cells. A low but unacceptable amount of non-specific binding occurred which became less frequent after using virus grown in Vero cells.

Immobilization of Antigen and other proteins: Rubella virions (or other proteins) were diluted in carbonate-bicarbonate buffer, 0.01 mol/L, pH 9.6, and dispensed in 50 ul portions to 96 wells of microtiter plates (type M129A, flat-bottom, Dynatech Labs, Alexandria, VA. 22314 or Linbro, flat-bottom, Flow Laboratories, McLean A. 22102) and desiccated under vacuum. This process causes a large and reproducible amount of applied protein to adsorb to the well surface as determined by ^{125}I-labeled protein. The dried virus was stable for at least 3 months if kept in sealed plastic bags in the dark at room temperature. In contrast, adsorbed immunoglobulins were stable for only 3 to 4 days even at 5°C.

Control Antigen: Because of the high degree of purity of the rubella virions we generally experienced no difficulty from nonspecific binding which would result in a false positive result. However, some culture fluid elements may become integrated with the virus coat and some sera appeared to contain antibodies to such elements. Most satisfactory control antigen among several tested consisted of 3 parts sonicated, particle free vero cell protein to 1 part fetal calf serum. When 400 ng (6) of this mixture was immobilized (by desiccation) a solid-phase antigen condition was obtained for absorbing out the offending antibodies before final

incubation with rubella antigen. See Table 2 for results comparing corrections for non-specific binding by 1) subtraction of control antigen absorbance (Abs_C) from absorbance with rubella antigen (Abs_R) to give incorrect net value (i.e. Abs_R minus Abs_C = Net) in contrast to 2) absorbing out the improper antibodies during a preliminary incubation before final incubation with rubella.

Antisera. One antiserum having antibody activity primarily to human IgG but also to IgM and IgA and a second antiserum having activity only toward IgM were prepared in house. These antibody activities were raised by four weekly subcutaneous injections of 100 micrograms of the appropriate antigens in complete Freund's adjuvant into four sites on the rump and back of each goat. Blood was collected for testing by Ouchterlony analysis on day 28 and at biweekly intervals thereafter before booster injections until antibody levels reached an equivalence titer of 1:8 when serum was harvested. The IgG fraction containing antibodies was isolated using octanoic acid and diethyl aminoethyl cellulose (DEAE) (7). The antiserum to IgM was further subjected to three 18 h absorptions with solid phase pooled human IgG (8) to remove all traces of anti-IgG. (Table 3). The IgG fractions of two additional antisera prepared in goats were obtained from Cappell Laboratories, Cochranville, PA. 19330. One was directed toward the Fab fragment of human IgG and the second was directed to the Fc component of human IgG.

Conjugation of enzyme to IgG-containing antibody.

Conjugates to glucose oxidase were prepared by a modification of the method of Wilson and Nakane (9). Briefly, glucose oxidase

TABLE 2.

Use of Control Antigen to Correct Nonspecific Absorption
See text for details.

Sample	HIA titer	Absorption at 410 nm			
		Antigen in Wells		Net Value	Bound to rubella after prior absorption
		Rubella	300ng Vero 100ng Calf Serum		
17852	< 8	0.277	0.105	0.172	0.232
00379	< 8	0.376	0.150	0.226	0.301
9081	< 8	0.290	0.100	0.190	0.294
17345	< 8	0.312	0.136	0.176	0.197
22823	1:16	0.528	0.219	0.309	0.373
31823	1:32	0.714	0.243	0.471	0.662
Gibco Neg	< 8	0.324	0.120	0.204	0.298
Gibco Hi	1:256	0.870	0.680	0.190	0.808
Flow Neg	< 8	0.190	0.116	0.070	0.153
Flow Hi	~1:64	0.599	0.105	0.494	0.640

TABLE 3.

Specificities of the enzyme-coupled anti-sera toward human IgG and IgM. The results are given in absorbancies at 642 nm* caused by the enzyme coupled antisera which became bound toward immobilized sets of pure specific immunoglobulin.

a. Binding of Glucose oxidase-goat anti human IgG. (1:200 dil.).

	Zero ng	400 ng	200 ng	100 ng	50 ng	25 ng
To Human IgG #	0.014	0.611	0.600	0.454	0.234	0.104
To Human IgA o	0.039	0.583	0.530	0.319	0.113	0.040
To Human IgM o	0.046	0.319	0.150	0.083	0.063	0.055

b. Binding of Glucose oxidase-goat anti human IgM (1:500 dil.).

	Zero ng	400 ng	200 ng	100 ng	50 ng	25 ng
To Human IgM o	0.037	0.759	0.675	0.334	0.145	0.080
To Human IgG #	0.017	0.023	0.019	0.021	0.018	0.019
To Human IgA o	0.021	0.027	0.026	0.022	0.025	0.023

* The colored product of ABTS^R has two absorption maxima: 410-417 nm and 642 nm. We used 642 nm early in the work and later adopted readings at 410-417 nm because of the availability of these wave lengths on the commercially available plate readers.

\# Isolated from pooled human serum by use of octanoic acid and DEAE(7).

o Gifts from Hans Spiegelberg.

was reacted with potassium periodate, 0.04 mol/L, for 20 min, 20°C to produce aldehyde groups. Addition of the IgG containing antibody and pH adjustment to 9.6 produced Schiff base-type conjugates which were converted to strong covalent bonds by borohydride in a third step. The final product was preserved with an equal volume of glycerol. All conjugates were titrated versus a set of decreasing antigen-coated wells to determine best useable

dilution, conformity of response, and specificity. See Table 3 for example.

Glucose Oxidase. Glucose oxidase (GO_x) is a stable nonmammalian enzyme. We expose its presence using a coupled enzymic reaction:

$$1)\ \text{Glucose} + O_2 \xrightarrow{GO_x} H_2O_2 + \text{glucuronic acid}$$

then,

$$2)\ H_2O_2 + \text{ABTS} \xrightarrow{\text{Peroxidase}} \text{Color} + H_2O$$

Chemicals and special equipment. We obtained 2,2'-Azino-di-[3-ethyl-benzthiazoline sulfate(6)] diammonium salt (also called ABTS^R) from Boehringer-Mannheim, Indianapolis, IN. 46250, and horseradish peroxidase grade VI from Sigma Chemical Co., St. Louis, MO. 63178. All other chemicals were analytical grade or the purest obtainable. We used 300 µl-capacity microcuvettes in a spectrophotometer-240 from Gilford Instrument Laboratories, Oberlin, OH. 44074. Fluid in the plate wells was agitated by use of an "Aliquot Mixer" from Miles/Ames, Elkhart, IN 46515.

Separation of IgG from IgM. Serum IgM was isolated from IgG by use of sucrose gradient sedimentation (10) to validate the majority of separations by ion-exchange chromatography (11). Fractions 2,3, and 4 from the sucrose gradients were assayed separately, undiluted, and without dialyzing away the sugar. Fraction 4 routinely showed the greatest amount of IgM antibody. The 2 ml eluates from the columns of quaternary amino-ethyl cellulose (11) were 1:20 dilutions of the original serum. Before assay we brought the pH of each 1 ml portion of eluate to 7 by adding 60 to 70 mg crystalline K_2HPO_4-$3H_2O$.

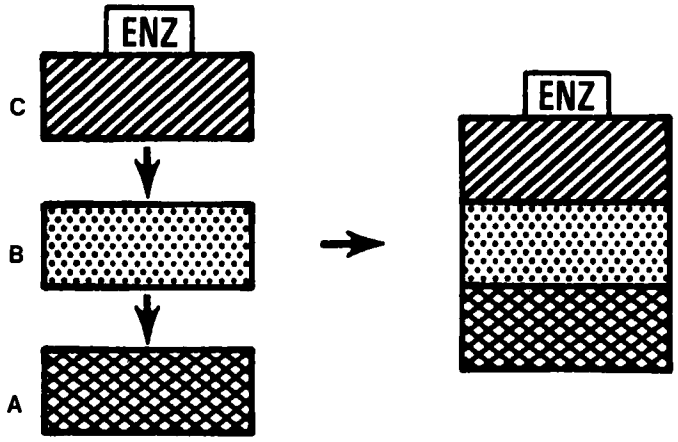

FIGURE 1. Diagram showing formation of the "sandwich" which is the basis of solid-phase EIA. "B" is always the element being measured and can be human immunoglobulin binding as antibody to "A", the rubella virus, coating the well. The second antibody, "C", binds enzyme in proportion to "B" to effect an absorbance related to the amount of "B".

In a variant assay, "B" can be known amounts of human immunoglobulin (IgG or IgM) which are added to become bound to a well coating, "A", of non-human antiserum specific for IgG or IgM. The "C", which can be identical to the previous, binds its enzyme again in proportion to "B", these known amounts of human Ig to allow construction of standard curves (mass of Ig vs Absorbance). Thus, the absorbancies obtained in the assay for antibody (here for rubella) can be translated into mg immunoglobulin equivalents per L-serum.

Standard Curves for IgG and IgM quantitation.

In this EIA for rubella antibody, a variant of that assay is used to obtain correspondence between absorbance and amount of bound first antibody (Fig. 1). The variant EIA uses specific antisera coated to wells to bind the known amounts of immunoglobulin on which the glucose oxidase-coupled second antibody

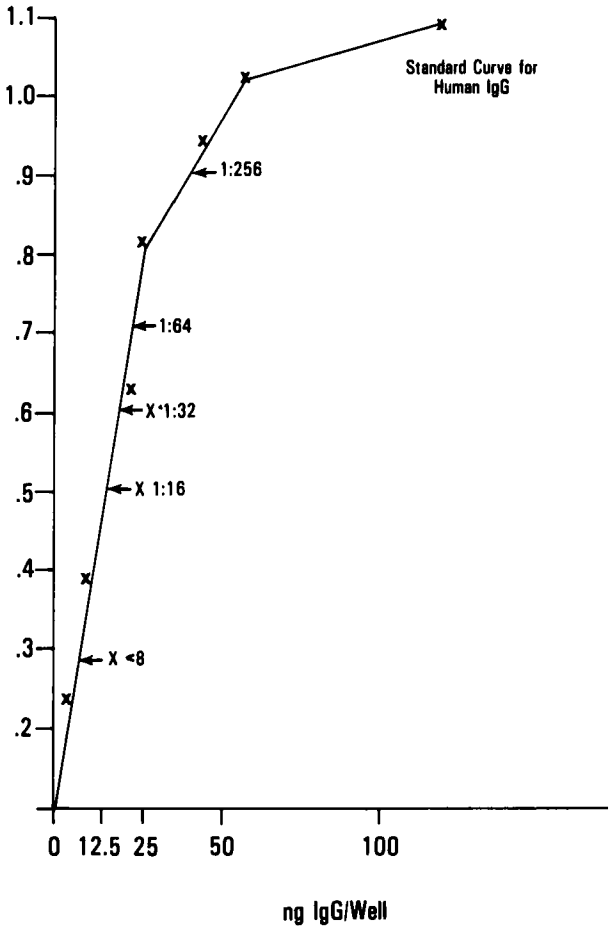

Figure 2. Standard curve for quantitating human IgG. Prepared by using immobilized antibody to trap known amounts of IgG from commercial standard serum and pure pooled IgG. Arrows indicate the mean absorbancies for each category grouped by HAI titer (Figure 4). Note, only nanogram amounts produce the entire curve and each adjacent HAI titer is separated by a mere 5 nanograms of bound IgG-equivalent. Use of this curve was validated by comparing the results on 12 sera with those obtained by radial immunodiffusion ($r = 0.95$).

binds. Commercial sera provided as immunoglobulin standards for the radial immunodiffusion (RID) assay of human sera (Meloy Laboratories, Inc., Springfield, VA. 22151) were one source of immunoglobulin standards. Human IgG isolated from pooled serum by octanoic acid and DEAE-treatment (7) then assayed by method of Lowry et al (6) was an equivalent source for IgG. IgG was trapped and bound by anti-Fc human IgG prepared in goats (4 mcg goat IgG per well). A conjugate of glucose oxidase with anti-Fab fragment of human IgG prepared in goat and comparable in action to the GO_x-anti-IgG H & L used in the rubella assay completed the reagents used for the IgG standard curve. (Figure 2).

The IgM curve was prepared by binding IgM from dilutions of standard sera to anti-IgM (3.5 mcg goat IgG per well) of the same lot from which the glucose oxidase conjugate was prepared. Here absolute equivalence occurred between the rubella antibody and standard curve absorbancies. The multiplicity of antigens on IgM and the "polyvalence" of the antiserum compared to IgG made this possible. (Figure 3).

Special Solutions. We used a diluting-wash solution (PBS/T/BSA) composed of bovine serum albumin 5 g/L and Tween-20 [polyoxyethylene (20) sorbitan monolaurate], 5 g/L in PBS at pH 7.2. A second wash solution (PBS/T) was identical to the PBS/T/BSA but lacked bovine albumin. Both solutions contained sodium azide, 1 g/L. Buffer-substrate was prepared as needed by combining 120 volumes phosphate buffer, (0.1 mol/L, pH6) with 1 volume ABTS (20 g/L H_2O), 1 volume peroxidase (1 g/L H_2O), and 15 volumes glucose (180 g/L in water and several hours old).

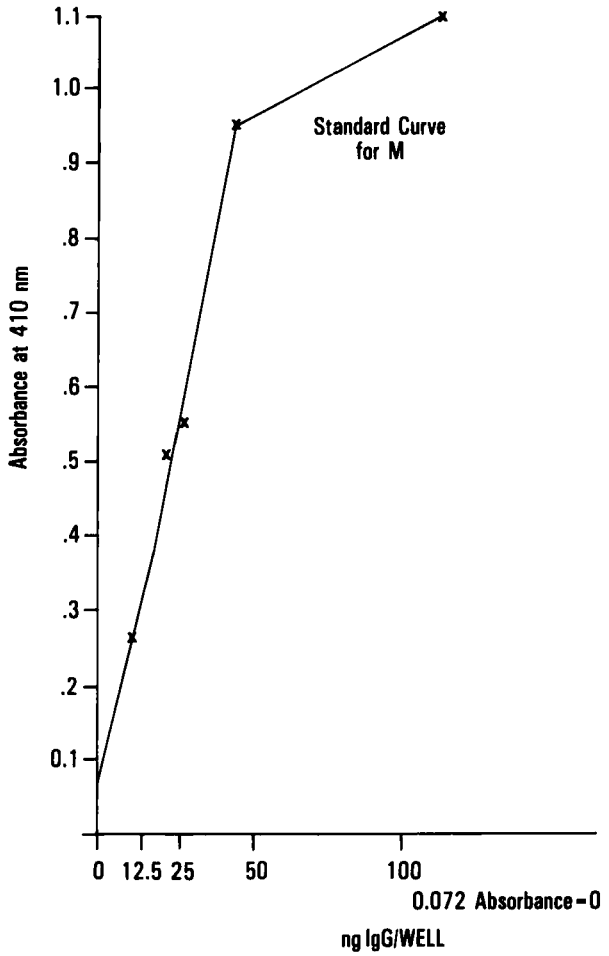

Figure 3. Standard curve for quantitating human IgM. Use of this curve was validated by comparing results on 12 sera with those by radial immunodiffusion (r = 0.94).

Preparation and dilution of sera: All samples were kept frozen (-60°C) until used. Sera were not inactivated. Both control sera and sera to be assayed were diluted equally in PBS/T/BSA. Early assays were performed using 1:64 dilution but 1:100 was found more

satisfacory. Sera were diluted just before assay and also 18 hours earlier in some instances.

EIA Protocol

A 96-well plate coated only with rubella provides for testing 41 sera in duplicate and a set of 3 control sera (low positive, high positive and negative) in quadruplicate (2 sets in duplicate). At time of assay we pretreated the rubella-coated wells to obtain enhanced antigenicity by filling with a solution of 20% acetone in normal saline allowing to stand 30 minutes at room temperature before removing by flicking into a sink. We washed the wells 3 times, first with PBS/T/BSA and twice with PBS/T for 3 min each time, then made the wells semidry by striking inverted against toweling.

We added no sera to the first 2 wells, using these as controls to monitor any nonspecific binding by the enzyme conjugate to the virus alone. Successive duplicate wells received 50 ul of diluted test or control serum or undiluted, pH-adjusted eluate.

The wells, covered with Parafilm^{R} or a plastic cover, incubated on a rocker 16 to 18 hrs at room temperature (20^{o}C) then were washed 3 times as before with PBS/T/BSA and PBS/T.

Glucose oxidase-labeled second antibody diluted in PBS/T/BSA was added to all the semidry wells. This second antibody was usually anti-human IgG but antihuman IgM was added when wells contained IgM in column eluates or appropriate fractions from sucrose gradient sedimentation. This incubation was only one hour.

Finally the wells were washed three times, 3 minutes each with PBS/T.

Fresh prepared buffer-substrate (200 ul) was added to all wells. We did this as rapidly as possible if a plate reader was used; alternatively in the absence of a plate reader, additions to successive duplicate wells were spaced at 15 second intervals. Incubation was always 30 minutes at room temperature (ca 20°C). In the absence of a plate reader we transferred and pooled the fluid from duplicate wells into clean test tubes also at 15 sec intervals. Enzymatic activity stops on transfer. We read the absorbancies at 410, 414, or 417 nm whichever was available vs H_2O or the blank control.

The positive and negative standard sera assayed along with the test samples have pre-established concentrations of rubella antibody in HAI titer and in mg IgG equivalents per liter serum. These values were obtained by multiple appropriate testing.

Hemagglutination Inhibition (HIA) Assay: The procedure used followed the heparin-$MnCl_2$ serum treatment and method described by the Center for Disease Control of the United States Department of Health, Education and Welfare, Atlanta, Georgia (4). This was further modified by including an additional absorption by chick erythrocytes at 56°C as described by Cremer (5) prior to actual testing.

RESULTS

Qualitative Agreement of SP-EIA and HAI.

One hour incubation, 20°C, with serum and rubella antigen produced complete agreement only when antibody levels were >1:32 by HAI (190 sera). Similar results have been given by earlier

TABLE 4.

Effect of long (18 h.) vs short (1 h.) incubation time on amount antibody bound to antigen.

Sample No.	HIA Titer	Absorbancies and Interpretation After 1 h Incub.	After 16-18 h Incub.
15512	1:16	0.268 Neg.	0.423 Neg[a]
16828	1:16	0.382 Neg.	0.684 Pos.
18540	1:16	0.407 Neg.	0.634 Pos.
20389	1:16	0.187 Neg.	0.462 Indeterm.[b]
31636	1:16	0.154 Neg.	0.359 Neg.[b]
9181	1:16	0.340 Neg.	0.635 Pos.
32196	1:32	0.350 Neg.	0.631 Pos.
32195	1:32	0.273 Neg.	0.559 Pos.
32109	1:32	0.380 Neg.	0.623 Pos.
31484	1:32	0.305 Neg.	0.653 Pos.
28861	1:32	0.252 Neg.	0.537 Pos.
27348	1:32	0.329 Neg.	0.547 Pos.
27599	1:32	0.245 Neg.	0.550 Pos.
28860	1:32	0.379 Neg.	0.678 Pos.
2678	1:32	0.332 Neg.	0.613 Pos.
7829	1:32	0.275 Neg.	0.557 Pos.
12355	1:32	0.433 Neg.	0.623 Pos.
Gibco Neg Control		0.437 --	0.452 -

a. Assays by indirect fluorescent antibody were negative but positive by passive HAI

b. Assays by indirect fluorescent antibody and passive HAI were < 1:8 and negative, respectively

investigators (12-15). Interestingly, the single serum with HAI titer of 1:8 showed presence of antibody under these conditions.

Increasing the incubation time to 16 to 18 h, 20°C, allowed sera weakly positive by HAI to become positive by SP-EIA. Now

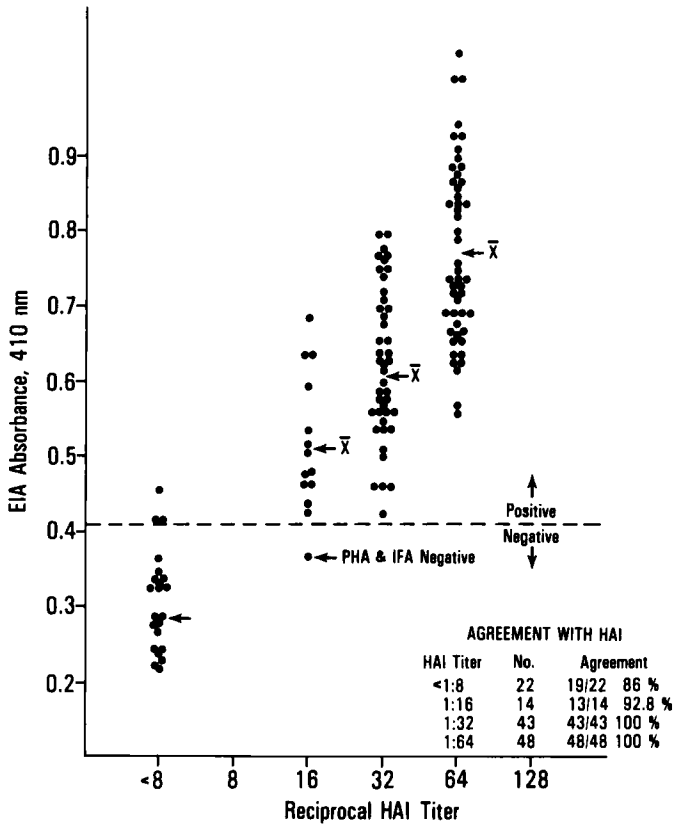

Figure 4. Comparison of rubella antibody titers tested by HAI and EIA. Arrows indicate the mean absorbance (410 nm) for each category grouped according to HAI result. Note the regular progression of these means with increase in titer. Note also the spread of absorbancies among each group and consider the inexactness of HAI titers. All EIA results here are from 16 to 18 h initial incubation to bind rubella antibody to immobilized antigen.

complete agreement occurred with sera showing HAI titers >1:16 ($^{123}/_{127}$ sera) and enabled 14 of 17 sera with low antibody levels by HAI to have increased absorbances in the positive range (Table 4). The agreement is 97% for 127 sera. Three sera with HAI titers <1:8 but yielding absorbancies above the negative range were retested after absorption by control antigen. All had new absorbancies in the negative range. Sera found negative by SP-EIA but having antibody by HAI were retested using passive hemagglutination (PHA) and indirect immunofluorescent antibody assay (IFA). Two sera were negative by both tests; one was negative only by IFA (Table 4). These studies offer strong support for the reliability of SP-EIA.

Quantitative Agreement of SP-EIA and HAI.

The 16 to 18 h incubation caused each set of sera classified by HAI titer to yield an average absorbancy that increased about 0.1 unit with each succeeding increase in HAI titer (Figure 4). This result demonstrates the ability of SP-EIA to indicate small concentration changes over a critical range of antibody concentration.

The SP-EIA absorbancy ranges of each set of sera classified by HAI titer overlap the absorbancy range of a neighbor (Figure 4). This overlapping mirrors the fact that HAI titers are reproducible only within plus/minus one doubling dilution. In contrast, the SP-EIA has a reproducibility of about $\pm$0.025 absorbance.

Assay of IgM Antibody: Ion exchange (11) and sucrose gradient sedimentation (10) were compared as means to isolate serum IgM from

IgG. The results on six sets acute and convalescent sera from patients with documented rubella infection confirmed the efficiency of ion-exchange. (Table 5). Even though a certain excess of rubella antigen is provided, removal of virtually all competition by IgG-type antibody insures optimal conditions for binding by any IgM antibody if present. Interference by rheumatoid factor is also avoided.

HAI Titer Equivalents and mg IgG and IgM Equivalent/titer.

Absorbancies have been shown to be proportional to rubella antibody level. Absorbancies produced by sera of defined HAI titer show a direct relationship (Figure 4) which can be defined by a standard curve to enable absorbancies given by newly assayed sera to be translated into approximate HAI-titer equivalents. But such expressions of concentration indicate discontinuity and are therefore inexact because antibody concentrations vary continuously among a random set of sera. SP-EIA expresses this continuum by giving an uninterrupted series of absorbancies (Figure 4). For this condition, concentration is best expressed as some mass of antibody per unit volume. Because stable IgG and IgM rubella antibody standards of defined mass have limited availability, IgG and IgM of unspecified antibody activity and widely available in defined concentration can serve as surrogate standards when defined amounts are made solid-phase. The reasonableness of this approach is shown in Figure 1. Standard curves relating mass of Ig bound to absorbance generated are shown in Figures 2 and 3. Using these standard curves allows absorbancies in the SP-EIA for rubella to be

TABLE 5.

Comparison of Sucrose Gradient and Ion Exchange as a means to isolate IgM-rubella antibody. All sera were from patients positive for rubella.

Serum No.	Days after onset	A_{410}^{nm} due to IgM bound to rubella: Sucrose Gradient (10) Fraction 4	Ion Exchange (11) 1:20 dilution, pH 7
1	1	0.447 (1.16[a])	0.436
2	14	0.778 (1.15)	0.612
3	16	0.988 (1.15)	0.865
4	1	0.356 (1.15)	0.362
5	32	0.586 (1.13)	0.460
6	2	0.841 (1.15)	0.774

[a]Indicates the density (g/cm^3) of sucrose solution of fraction taken for assay

translated into mg IgG (or IgM) - equivalents per L. Such treatment was given the 10 sera with results reported in Table 6. The calculation to translate absorbancy to mg-Ig-equivalent/L-serum is: ngIg found from absorbance curve x 0.02 x serum dilution.

DISCUSSION

A method to assay rubella antibody by solid-phase enzyme immunoassay has been presented that meets many virtues not found in earlier methods. First, the method is simpler. Only one dilution of serum is required in nearly all cases to determine both presence

TABLE 6

Paired Rubella-positive Sera Tested for IgG and IgM Antibodies

Spec. #	Days after onset	HAI titer[a]	Abs 410 nm for IgG	mg IgG-equiv per liter	Abs 410 nm for IgM	mg IgM-equiv per liter
594a	0	<8	0.440	25	0.436	30
594b	14	256	0.853	60	0.612	52
595a	2	<8	0.476	27	N.D.	N.D.
595b	16	128	1.077	200	0.865	74
596a	1	<8	0.347	20	0.362	37
596b	32	128	1.015	120	0.460	42
649a	2	<8	0.585	30	0.774	68
649b	16	256	0.860	60	0.699	58
710a	2	<8	0.528	26	0.368	37
710b	16	256	0.932	96	0.482	44

a. Results expressed as HAI equivalents.

N.D. = Not Done

and quantity of antibody. Differences in antibody level between two sera - as for example an acute phase sample and one at convalescence - can easily and reliably be determined. This quality of the method is obtained through the contrived use of adequate excess rubella antigen and also a sufficiency of enzyme-labeled second antibody so that these components are not restricting the expression of any rubella antibody concentration up to that showing a 1:256 titer by HAI. The method is simpler also because testing against a control antigen is seldom required due to the high purity of the rubella antigen itself. More shall be said concerning these antigens.

Second, the method is accurate in defining amount of antibody. Thus the absorbancies correlate well with titers of antibody found by HAI. This quality of the method depends on the previous conditions but now refined to include 1) adequate incubation time to permit binding by the full spectrum of antibodies to the entire array of antigens, 2) a purified antigen that shows minimal affinity to the second antibody, and 3) a second antibody coupled to glucose oxidase such that neither non-specific binding nor endogenous enzymatically produced absorbancies occur to mar the responsive immune reaction which causes active enzyme to become solid phase and produce absorbancies in proportion to the amount of rubella antibody previously made solid phase.

Third, the method shows a high degree of reliability for indicating presence of antibody by showing overall a 97% concordance with the standard hemagglutination inhibition assay. Complete agreement between these two assays occurs among sera

having antibody concentrations >1:16 by HAI. SP-EIA showed 86% agreement for those sera with titers <1:8 through 1:16 by HAI. Several discordant sera of this group when retested by passive hemagglutination (PHA) and indirect immunofluorescent antibody assay gave results with one or both assays agreeing with the results found by SP-EIA. Control antigen was only found useful if used in a preliminary step to absorb nonspecific antibodies before testing against rubella antigen alone. The procedure is time consuming, requires special control antigen adsorbed to wells, and sera requiring such treatment seem infrequent. These sera show absorbancies in a borderline area. Perhaps such sera should be regarded as having an indeterminate result by SP-EIA and be retested by passive hemagglutination and/or indirect immunofluorescent antibody for clarification. More recently (16), however, it has been reported that a few individuals with low HAI titers of antibody become viremic following challenge with intranasal RA 27/3. These findings emphasize Sever's mandate (17) to save all sera for 1 year so repeat testing may be conducted.

Fourth, the method identifies IgM antibody easily and reliably by incorporating a simple preliminary ion-exchange procedure to remove both any competing IgG antibodies and any IgM-type rheumatoid factor interference. Sucrose gradient and ion exchange separation appear equivalent. Use of a special glucose oxidase labeled antibody specific for mu chain increases the reliability.

Fifth, the method quantifies antibody into HAI-titer equivalents or into milligrams of IgG (or IgM) - equivalents per liter. The HAI-titer equivalent is proposed to be an interim

unitage to provide means to acquaint physicians with interpretation of the milligram IgG (or IgM)-equivalent. The proposal to describe antibody concentration in amounts of IgM is not new with this report. Leinikki et al (2) had described use of affinity chromatography-purified rubella antibody as a standard to obtain the relationship between mass of antibody and absorbance. Because such purified antibody is not widely available we here introduce the use of IgG and IgM as classes of identifiable proteins that can act as surrogate standards in SP-EIA. Benefit occurs because standardized amounts of these are widely available and the second antibody which causes the enzyme to become solid phase can not distinguish any difference between the surrogate standard and that type rubella antibody. Difference does come in the results given by Leinikki and this proposed new method. Thus, the former method indicated sera with HAI-titers <1:8 to have no more than 1 mg IgG/L and in contrast we find such sera to have up to 30 mg/IgG-equivalents/L. We defend our results by these observations: 1) sera with HAI titers <1:8 as a group have an average absorbance about 0.3 with a range of 0.2 to .45 (Figure 4); 2) pre-treatment of a serum by absorption with control antigen substances does not reduce the absorption given by negative sera below our detection limit of 6 mg IgG/L serum diluted 1:100. (See Flow negative and Gibco negative, Table 2). 3) The standard curves for IgG and IgM were proved valid by showing their use in quantifying those immunoglobulins in sera to obtain results with correlation coefficients of 0.94 and better in comparison to results by radial

immunodiffusion; 4) the results here are described as equivalent not as specific antibody.

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