Enzyme-Linked Immunosorbent Assay Determination of Specific Rubella Antibody Levels in Micrograms of Immunoglobulin G per Milliliter of Serum in Clinical Samples

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A "microgram assay" is described in which solid-phase enzyme-linked immunosorbent assay is used for the determination of specific rubella immunoglobulin G (IgG) antibody levels in micrograms per milliliter of serum. The quantitation was based on a standard curve obtained by using a reference serum, for which the specific IgG content was assayed by immunochemical purification. IgG was first purified and specific rubella antibodies were separated by an immunoadsorbent prepared by linking rubella virus antigens to Sepharose 4B. By using IgG-specific conjugate, the levels of specific rubella IgG antibodies could then be determined from clinical samples. Seronegative samples showed antibody levels less than 1 $\mu\text{g/ml}$, whereas levels up to several hundred micrograms per milliliter were detected in some postinfection sera. The correlation between microgram antibody levels and hemagglutination inhibition titers was linear. The method offers a simple and sensitive antibody assay which could be used both for the laboratory diagnosis of acute rubella and for the evaluation of immunity.

Solid-phase enzyme-linked immunosorbent assay (ELISA) has gained wide interest as a potential new method to replace conventional antibody assays in the laboratory diagnosis of viral infections. It offers good sensitivity and reproducibility, stability of reagents, and simple technical performance. A number of variations in the performance of the ELISA have been reported (3, 6, 7), and methods for quantitating antibodies differ among investigators (4, 8, 9). In addition, accurate correlation of ELISA results with conventional antibody titers has not been established. As the ELISA becomes increasingly used for clinical and research virology, it is important that a precise technique be established for determining the correlation between test readings and actual levels of specific antibody.

An ELISA method applicable for routine and research virus serology was recently described (3). The quantitation was based on the use of a standard serum, and the optical densities from the serum samples could be converted to relative amounts of antibody through a simple program directly applied to the photometer.

We describe here an application of this technique to the determination of virus-specific immunoglobulin G (IgG) antibody levels expressed in micrograms per milliliter of serum. This "mi-

crogram assay" is simple to perform, only small sample volumes are required, and it can be used with clinical specimens. The microgram levels of rubella antibodies were found to show a linear correlation with hemagglutination inhibition (HI) antibody titers in postinfection sera. The use of generally available reference sera will aid in the standardization of the assay in different laboratories.

MATERIALS AND METHODS

Rubella virus antigen. The details of the technique used for propagating rubella virus for the ELISA have been published recently (2). Briefly, concentrated culture medium from infected Vero cells was centrifuged in a sucrose gradient, and the virion band was collected, dispensed in appropriate working volumes, and stored at -70°C . For the test, cuvettes were sensitized with this preparation diluted in phosphate-buffered saline (PBS), pH 7.4. The working dilution for the antigen (usually 1:100 to 1:200) corresponded to approximately four antigenic units, as measured by sensitizing cuvettes with serial dilutions of the antigen and determining the end point by using known positive serum.

Serum samples. Paired and single sera were available from two outbreaks of rubella through the courtesy of L. Mayhall, Medical College of Virginia, Richmond, and J. H. Barrick, Department of Public Health, Division of Laboratory Services, Nashville, Tenn. Sera from laboratory personnel and clinical patients were also available.

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Preparation of anti-human IgG-alkaline phosphatase conjugate. Commercially available swine anti-human IgG rendered heavy chain-specific by solid-phase immunoadsorptions (Orion Diagnostica, Mankkaa, Finland) was further purified by applying it to an immunoadsorbent column prepared by coupling cyanogen-bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) with human immunoglobulin (5; see below). To about 5 ml of immunoadsorbent gel in a small column 1 ml of anti-human IgG was applied. After washing with PBS, the specific immunoglobulins were eluted by glycine-hydrochloride buffer (0.1 M, pH 2.5). As soon as the protein came out, peak fractions were pooled, the pH was adjusted to 8 by 0.2 M tris(hydroxymethyl)aminomethane buffer, and the volume was concentrated to 0.2 ml. The amount of specific anti-human IgG obtained in each purification was approximately 1 mg. The anti-human IgG and alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.) were then conjugated by using glutaraldehyde as described by Engvall and Perlman (1). The working dilution of the conjugate was assayed by using cuvettes sensitized with 10 μ g of human IgG per ml in carbonate buffer, pH 9.5. The working dilution was chosen close to the point of equivalence (3). Recently, the activity has been monitored by comparing the activities of various lots of conjugates in rubella-sensitized cuvettes reacted with known amounts of specific anti-rubella IgG.

ELISA for rubella virus antibodies. The principles of the performance of the test and the quantitation of the results have been described previously (3, 4). In brief, disposable polystyrene cuvettes (Finnpipette-Labsystems, Helsinki, Finland) in blocks of nine were sensitized by incubating 200 μ l of antigen at the working dilution in PBS overnight at 4°C. The cuvettes were then washed two times with PBS-0.5% Tween 20 and rinsed with distilled water two times. Serum samples diluted 1:50, 1:500, and 1:5,000 with PBS containing 0.05 M ethylenediaminetetraacetic acid and 1% bovine serum albumin were added (200 μ l/cuvette), incubated at 37°C for 90 min, and washed as above. Conjugate (200 μ l/cuvette), diluted in the same diluent as the serum samples, was then incubated for 90 min at 37°C, and the excess was washed away as described above. Finally, 200 μ l of substrate (*p*-nitrophenyl phosphate, Sigma 104) at 1 mg/ml in diethanolamine buffer, pH 10 (8) was added. The color reaction was stopped after a 30-min incubation at 37°C by adding 400 μ l of 1.5 M NaOH. The color was measured by using a nine-channel photometer (model FP9; Finnpipette-Labsystems) which measures the optical densities vertically through the bottoms of the sensitized cuvettes. Each reading was compared with a reference curve prepared from a serum which had a known amount of rubella IgG antibodies determined as described below. The photometer is equipped with a Hewlett-Packard HP9815A calculator which can be programmed to give the direct conversion of optical densities to micrograms per milliliter.

Preparation of IgG. Immunoglobulins from the reference serum were precipitated by one-third-saturated ammonium sulphate and dialyzed against 0.01 M phosphate buffer, pH 7.6. This solution was sub-

jected to ion-exchange chromatography on DE-52 (Whatman, Kent, England). The peak eluted with 0.01 M phosphate buffer was then collected, concentrated, and dialyzed against PBS overnight at 4°C. This IgG preparation was then purified by using a rubella-specific immunoadsorbent (see below).

Preparation of rubella virus immunoadsorbent. Rubella virus (250 to 300 μ g) was disrupted by adding Nonidet P-40 to a concentration of 5% (vol/vol), after which the mixture was incubated at room temperature for 2 h. A 2-g quantity of cyanogen-bromide-activated Sepharose 4B was washed with dilute acid and mixed with the viral proteins. The Sepharose-protein mixture was gently stirred for 2 h at room temperature and then placed in a small column. In the column the immunoadsorbent was washed with PBS, 2 M urea in PBS, 0.1 M glycine-hydrochloride, pH 2.5, PBS-0.05 M ethanolamine, and finally with PBS. The immunoadsorbent was stored at 4°C and could be used repeatedly.

RESULTS

Separation and quantitation of specific rubella virus IgG antibodies in the reference serum. IgG antibodies were purified from 5 ml of reference serum by ammonium sulphate precipitation and ion exchange chromatography (see above). The reference serum was from a healthy individual and had a high rubella virus HI titer. From the IgG preparation specific rubella IgG antibodies were separated by adsorption and elution with rubella virus immunoadsorbent (Fig. 1). The immunoglobulin solution was applied to the immunoadsorbent in a small column. The gel was washed thoroughly with PBS, and, after no more protein was detectable in the eluate, glycine-hydrochloride buffer (0.1 M, pH 2.5) was added to elute the specific antibodies. The peak fractions were pooled and immediately neutralized with 0.2 M tris(hydroxymethyl)aminomethane. In the experiment shown in Fig. 1, the actual protein concentration of the peak was 55 μ g/ml, as measured by optical density against a known standard. When this and the original serum were compared in the ELISA, the purified preparation had anti-rubella activity which was 0.8 log less than that of the original serum. Thus, the actual amount of specific IgG in the reference serum was equal to the antilog of $\log 1.73 + 0.8 = 2.53$, which is 340 (Fig. 2). Other lots gave comparable results.

Rubella IgG antibody levels in serum samples and their correlation with HI titers. Seven preinfection sera with no detectable HI antibodies showed antibody levels of less than 1 μ g/ml. Early acute-phase samples from 12 rubella patients showed levels of antibodies of from 1 to 20 μ g/ml, from which the levels increased to 20 to 251 μ g/ml in convalescent-phase sera taken after 2 to 4 weeks (Table 1).

FIG. 1. Purification of rubella-specific IgG antibodies. The immunoadsorbent was prepared by coupling cyanogen-bromide-activated Sepharose 4B with sucrose gradient-purified virions disrupted with 5% Nonidet P-40. In a small column (0.9 by 15 cm) the gel (5 ml) was washed with 2 M urea, glycine-hydrochloride (0.1 M, pH 2.5), PBS-ethanolamine (0.05 M), and PBS. A 1-ml quantity of IgG solution in PBS (protein, 13.2 mg) prepared by ammonium sulphate precipitation and ion exchange chromatography was then applied, and the column was washed with PBS. Specific IgG was eluted with 0.1 M glycine-hydrochloride buffer, pH 2.5, collected, and neutralized with 0.2 M tris(hydroxymethyl)aminomethane. O.D. 280, Optical density at 280 nm.

FIG. 2. Determination of microgram levels of specific rubella IgG antibodies of the reference serum by using purified rubella IgG antibodies with a known protein content. Serial dilutions of the reference serum were assayed by ELISA (standard curve). Three dilutions (1:50, 1:500, and 1:5,000, corresponding to logs -1.7 , -2.7 , and -3.7 , respectively) of the immunoadsorbent-purified IgG antibody were similarly assayed. The difference between the dilutions of the two prepartes giving the same optical density at 45 nm (OD 405) (ED value) indicates the relative antibody content of the preparations. Because the amount of protein in the purified rubella IgG was known (55 $\mu\text{g}/\text{ml}$), the amount of IgG in the reference serum is the antilog of $\log 55 + \text{ED}$, which is $1.74 + 0.8$, which is 340.

A total of 28 late-convalescent-phase sera showed varying amounts of specific rubella IgG antibody levels, ranging from 2 to almost 400 $\mu\text{g}/\text{ml}$. These antibody levels showed a linear correlation with HI titers (Fig. 3). Because of this linear correlation normal values for IgG antibodies could be indicated. Levels between 4 and 40 $\mu\text{g}/\text{ml}$ could be regarded as normal in immune persons, because they correspond to HI titers of 16 to 128. Levels equal to or greater than 80 $\mu\text{g}/\text{ml}$ correspond to high HI titers and are most frequently seen in convalescent-phase sera. Levels equal to or less than 1 $\mu\text{g}/\text{ml}$ indicate lack of antibodies and lack of immunity. Interestingly, the early acute-phase samples (Table 1) did not show a completely linear correlation with HI titers. In several samples HI titers were higher than could be anticipated from the ELISA IgG levels. This was shown to be due to IgM antibodies which did increase the HI titers but did not affect the ELISA IgG levels (P. O. Leinikki et al., *J. Clin. Lab. Invest.*, in press).

DISCUSSION

In a previous publication the use of a standard curve for the correlation between optical densities and serum antibody levels was found to be essential for the quantitation of serum antibodies in the ELISA (4). We now report a microgram assay for determining specific microgram levels of IgG antibodies in serum specimens by using a reference serum for which the specific antibody level was determined by separation and immunochemical purification of rubella-specific IgG antibodies and determination of their specific activity.

TABLE 1. ELISA antibodies and HI titers in acute- and convalescent-phase serum samples from patients with rubella

Patient no.	IgG antibodies ($\mu\text{g}/\text{ml}$) in:		HI Titers in:	
	Acute-phase serum	Convalescent-phase serum	Acute-phase serum	Convalescent-phase serum
1	2.0	251.0	16	256
2	2.5	31.6	8	32
3	2.5	50.1	<4	64
4	1.0	63.0	<4	64
5	2.0	158.0	4	256
6	2.0	79.4	64	256
7	6.3	158.0	8	512
8	8.0	200.0	16	256
9	2.0	251.0	64	512
10	2.5	63.0	8	128
11	1.0	20.0	<4	256
12	1.0	31.6	<4	32

FIG. 3. Correlation between rubella virus ELISA antibodies (micrograms per milliliter of serum) and HI antibodies.

To be able to measure the actual levels of rubella IgG antibody in our reference serum, rubella-specific IgG molecules were separated from purified total IgG by adsorbing them onto a rubella virus immunoadsorbent in a column, and subsequently eluting them by lowering the pH. Low pH is known to cause some loss of antibody activity. In our assay this would lead to higher readings of micrograms per milliliter for the whole reference serum and, thus, for the samples. We minimized the loss of antibody activity by allowing only a short reaction time between the antigen and the antibody during absorption, thus facilitating the elution process, and also by immediately neutralizing the pH of the eluted IgG. Various lots from the same serum sample and other samples from the same individual showed little variation in activity, indicating a good recovery of the specific antibody. The technique is simple and allows other laboratories to make their own standards. Of course, an internationally available reference with known amounts of specific IgG antibodies would further simplify the standardization of the method.

Very high levels of rubella virus-specific IgG antibodies were found in some normal sera. For instance, in our reference serum, obtained from a healthy individual, approximately 1/70 of the total IgG was directed against rubella. By applying similar techniques to other antibody assays, it would be possible to map immunologically at least part of the immunoglobulins of an individual.

The cut-off value for seronegatives, 1 µg/ml, is higher than the absolute sensitivity of the test, which can be calculated from the reference serum. The greatest dilution of the serum that still gave an optical density reading significantly above the background corresponds to a serum dilution of 1:35,000. Thus, the corresponding amount of antibody would be 10 ng/ml. In samples which give less background than serum, e.g., cerebrospinal fluids, levels lower than 1 µg of specific antibody per ml can be reliably detected (P. O. Leinikki, unpublished data).

The good correlation between the observed IgG microgram levels and HI titers suggests that hemagglutinin plays an important role in the covering of the cuvettes during sensitization. Studies using similar cuvettes in another antibody assay also suggest that the sensitization is selective; although purified whole coronavirus virions were incubated in the plastic cuvettes, only one of the envelope proteins dominated the antigenic surface of the cuvette (P. O. Leinikki and K. Holmes, unpublished data).

The microgram assay also has direct clinical applications. The difference between seronegative samples and samples with low levels of antibodies was easy to determine, and it was sensitive in detecting increases in antibody levels during the disease. The postinfection levels of IgG antibodies of from 4 to 40 µg/ml corresponded to HI titers often seen in immune persons during late convalescence. Levels greater than 80 µg/ml should be regarded as high and are seen only for a relatively short time after the infection. Unlike HI titers, IgG levels detected by the ELISA are not affected by IgM rheumatoid factor. (Leinikki et al., in press).

The use of the described method for diagnostic antibody studies offers several definite advances. The reading of the results through a programmable photometer directly to micrograms per milliliter simplifies the interpretation of the results, both in the research laboratory and in the clinical laboratory. The accuracy achieved through the use of the standard curve may eliminate the need for including a previous sample from each individual when diagnostic rises in antibody levels are looked for. The rapid increase observed in the amounts of specific antibodies during the disease permits a serological diagnosis early in the disease. The technique also can be applied easily to other viral infections. We have recently used it for the assay of measles virus antibodies, and studies are in progress to apply it to the measurement of virus-specific IgM antibodies.

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