

# Changes in Antibody Avidity After Virus Infections: Detection by an Immunosorbent Assay in Which a Mild Protein-Denaturing Agent Is Employed

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**In titrating serum immunoglobulin G antibody to viruses by enzyme-linked immunosorbent assay, we used two rows of wells for serial twofold dilutions of the serum; in one row, a low concentration of a protein denaturant, 0.5 or 1.0 M guanidine hydrochloride, was added to the diluent so that the binding of low-avidity antibodies to viral antigens on the solid phase was inhibited. We then compared the antibody titration curves obtained in the two rows. We found that the addition of the reagent resulted in a parallel leftward shift of the curves and that the extent of the shift was greater in early than in late sera from all of the three infections studied (Japanese encephalitis virus, rotavirus, and rubella virus infections). This procedure may be useful for estimation of the avidity of antibody in serum and, with further evaluation, may prove to be applicable to single-serum diagnosis of virus infections.**

It is well known that the affinity or avidity of antibody (Ab) increases progressively with time after immunization of animals (6, 12, 20); this is the so-called maturation of the immune response (21). This phenomenon is considered to occur also in the immune responses of hosts after virus infections. Therefore, there may be a possibility of diagnosing a virus infection by measuring the avidity of immunoglobulin G (IgG) Ab in a single serum. Usually, the detection of IgG Ab to a virus in a serum is of no diagnostic value, since IgG Ab has a long persistence after virus infections. Detection of a rise in Ab titers between paired sera is required; the first serum specimen should be collected as early as possible after the infection. However, well-timed collection of paired sera is often an impracticable requirement. By determining whether the avidity of IgG Ab in a serum is low or high, one might be able to determine whether the serum was taken shortly or remotely after the infection.

However, the measurement of avidity of Ab to viruses is not easy. Since virus antigens are large in size with repeating antigenic determinants, the technique of equilibrium dialysis for hapten-Ab interactions (5) cannot be used, nor can a technique described by Farr (7) for determining the quality of Ab to soluble proteins which are not precipitable at 50% saturation of ammonium sulfate. Webster (22) studied the avidity change after immunization of rabbits with influenza viruses by the technique of equilibrium filtration. But this technique is time-consuming, laborious, and not suitable for routine diagnostic purposes. Recently, Lehtonen and Meurman (15) studied the avidity change after rubella infection by using an enzyme-linked immunosorbent assay (ELISA). In their ELISA system, they detected differences in the shapes of Ab titration curves between early and late sera taken after the infection.

We have now devised a new, simple ELISA procedure for estimating the relative Ab avidity, which may prove to be applicable for single-serum diagnosis of virus infections. In

this technique, a low concentration of protein denaturant is included in the serum diluent to prevent the binding of low-avidity Ab to the solid-phase antigens, and the Ab titration curves obtained in the presence of the reagent are compared with those obtained in its absence. This report describes the results in three kinds of virus infections: (i) experimental infection of guinea pigs with Japanese encephalitis (JE) virus, (ii) rotavirus infection of infants, and (iii) primary and secondary rubella infections.

## MATERIALS AND METHODS

**Sera.** For anti-JE sera, female guinea pigs were infected intraperitoneally with  $10^5$  50% lethal doses (determined by suckling mouse brain inoculation) of JE virus, JaGAR 01 strain (10). Serial serum samples were collected from the animals by heart puncture. For antirotavirus sera, sera were collected from infants with rotavirus gastroenteritis, for whom diagnosis was confirmed by detection of the virus in feces by electron microscopy or by a serological test (16). Rotavirus Ab-positive sera from healthy infants were also used as a control. For antirubella sera, paired sera from 14 rubella patients (see Table 3) and single sera from 8 patients (one male and seven females, ages 17 to 35) were used. Diagnosis of rubella in those patients was confirmed by detection of hemagglutination-inhibition (HI) Ab in 19S fractions after sucrose gradient centrifugation of the sera (8). Rubella Ab-positive sera from 35 healthy adults (5 males and 30 females, ages 20 to 35) were used as a control. HI Ab titers of these sera ranged from 1:16 to 1:256, with a mean of 1:64.

**Purified antigens for ELISA.** For JE virus, R66 cells were infected with JE virus, strain JaGAR 01, at a multiplicity of infection of 10 and maintained at 37°C for two days in Eagle minimal essential medium containing 0.1% bovine serum albumin. After the culture fluids were clarified by low-speed centrifugation, virions were concentrated by polyethylene glycol precipitation and then purified by isopycnic centrifugation in metrizamide density gradients (10). Virion fractions with complement-fixing antigen titers of 1:100 were used at a 1:300 dilution in the ELISA. For rotavirus, MA104 cells

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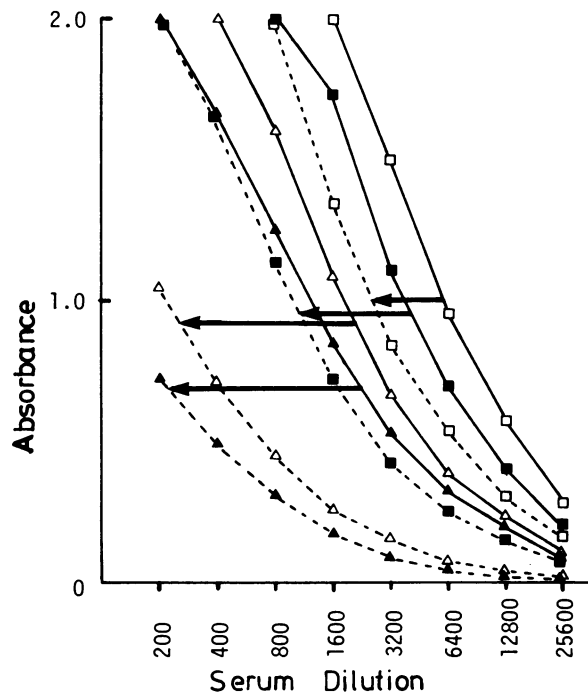


FIG. 1. Ab titration curves by the ELISA of sera sequentially collected from a guinea pig infected with JE virus. IgG Ab was titrated with (---) and without (—) 0.5 M guanidine hydrochloride in the serum diluent. Length of arrows indicates Ab dissociability, which was 3.4, 3.0, 2.0, and 1.3 on sera taken 2 weeks (▲), 3 weeks (△), 5 weeks (■), and 4 months (□), respectively, after infection.

were infected with human rotavirus, Wa strain, at a multiplicity of infection of 1.0 and maintained at 37°C for one day in minimal essential medium containing 2 µg of trypsin per ml. After cells were frozen and thawed twice, virus particles were concentrated by polyethylene glycol precipitation and then purified by isopycnic centrifugation in CsCl density gradients. The inner capsid band was harvested and treated with 2 M guanidine hydrochloride for fragmentation to enhance attachment to the microplate wells (11). The fragmented antigens were diluted to a final protein concentration of 1 µg per ml for the ELISA. Protein concentration was determined by the method of Bradford (3) with a commercially available kit (Bio-Rad Laboratories, Richmond, Calif.) and with bovine serum albumin as the standard. For rubella virus, BHK-21 cells were infected with rubella virus, M33 strain, at a multiplicity of infection of 1.0 and maintained at 37°C in minimal essential medium containing 0.5% calf serum. Culture fluids were replaced every day with fresh maintenance medium. The fluids harvested from 2 to 8 days postinfection were pooled and stored at 4°C. Virions were concentrated from the pool by the Pellicon Cassette System (Millipore Corp., Bedford, Mass.), further precipitated by 50% saturation with ammonium sulfate, and then purified by rate-zonal centrifugation in sucrose gradients (13). Virion fractions (hemagglutination titer of 1:32,000, protein concentration of 150 µg per ml) were used at a 1:1,000 dilution in the ELISA.

**ELISA procedures.** Purified antigens (see above) were diluted in 0.05 M carbonate buffer (pH 9.6), and 0.1-ml volumes were dispensed into wells of Immulon II Microelisa plates (Dynatech Laboratories, Inc., Alexandria, Va.) which

were then incubated overnight at 4°C. After the wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20, twofold serial dilutions (0.1 ml) of the serum from 1:200 to 1:25,600 were prepared in two rows of wells using 1% bovine serum albumin-phosphate-buffered saline with 0.05% Tween 20 with or without 0.5 M (in the case of JE and rubella viruses) or 1.0 M (for rotavirus) guanidine hydrochloride as the diluent. The plates were then shaken on a Micro-Mixer (MX-4; Sanko Junyaku Co., Tokyo, Japan) at room temperature for 2 h. After the wells were washed as described above, 0.1 ml of peroxidase-labeled, affinity-purified, rabbit Ab to guinea pig IgG (Zymed Co., Burlingame, Calif.) diluted 1:2,000 (in the case of JE virus) or goat Ab to human IgG (Tago Co., Burlingame, Calif.) diluted 1:3,000 (for rubella virus and rotavirus) in 1% bovine serum albumin-phosphate-buffered saline with 0.05% Tween 20 was added. The plates were shaken at room temperature for 1 h. The plates were then washed, and 0.1 ml of 0.012% hydrogen peroxide containing 0.04% orthophenylenediamine dihydrochloride in citric acid-phosphate buffer (pH 5.0) was added. After incubation at room temperature for 30 min without shaking, 0.05 ml of 2 M sulfuric acid was added to stop the enzyme action. Absorbance at 492 nm was measured by a Titertek Multiskan spectrophotometer (Flow Laboratories, Rockville, Md.). After Ab titration curves were drawn on graph paper, the horizontal distance between the two curves of each serum at an absorbance value of ca. 1.0 was measured (see Fig. 1, for example). The length (1 U = one doubling dilution) was defined as Ab dissociability of the serum.

**Other serological techniques.** Complement-fixation and immune adherence hemagglutination tests for rotavirus Ab and HI tests for rubella Ab were carried out as previously described (8, 9).

## RESULTS

**Experimental infection of guinea pigs with JE virus.** Ab titration curves of sera sequentially collected from a guinea pig are shown in Fig. 1. Titration curves were shifted leftward by inclusion of guanidine hydrochloride in the serum diluent. The extent of the shift (Ab dissociability, as defined above) was greatest (3.4) with serum taken 2 weeks after virus inoculation and then gradually diminished to 1.3 at month 4 postinfection. Thereafter, it remained at the same level for 1 year of observation (data not shown).

**Rotavirus infection of infants.** We then determined Ab dissociability of sera taken from patients with infantile gastroenteritis at various times after the onset of illness together with sera from healthy individuals (Table 1). It was found again that Ab dissociability decreased with time elapsed after infection.

**Primary and secondary rubella infections.** Ab dissociability was determined on paired sera from 14 patients and single sera from 8 patients with primary rubella infection (Table 2). The data on these sera (Fig. 2) show the relationship between Ab dissociability and days after onset of rash. Ab dissociability was found to be 1.5 or over within 1 month after the onset of illness. A gradual decrease in Ab dissociability, thus an increase in Ab avidity, after infection was again shown. For comparison, Ab dissociability of sera from 35 Ab-positive healthy adults (remote infection) was determined. Figure 3 shows the frequency in Ab dissociability of these sera; Ab dissociability ranged from 0.6 to 1.4, with a mode at 0.8. Therefore, Ab dissociability in recent infection was clearly separable from that in remote infection.

TABLE 1. Ab dissociability after rotavirus infection

Person no. <sup>a</sup>	Age	Days after onset of illness	Ab titer <sup>b</sup>		Ab dissociability
			CF	IAHA	
1	11 mo	12	256	<8	3.1
		34	128	512	1.7
2	11 mo	10	256	32	3.1
3	7 mo	10	128	64	3.3
4	6 yr, 1 mo	11	64	<8	3.7
5	1 yr, 3 mo	12	256	64	3.2
6	1 yr, 2 mo	22	256	1,024	2.3
7	1 yr, 1 mo	34	64	256	1.5
		53	32	128	1.0
		91	32	128	0.5
8	1 yr, 2 mo	54	128	512	1.2
		95	128	1,024	0.2
10	1 yr		NT <sup>c</sup>	128	0.3
11	2 yr		NT	256	0.6
12	6 yr		NT	128	0.5
13	25 yr		NT	256	0.2
14	2 yr		NT	64	0.6
15	3 yr		NT	128	0.8
16	4 yr		NT	128	0.7
17	9 mo		NT	256	0.5
18	4 yr		NT	128	0.3

<sup>a</sup> Person no. 1 to 9, rotavirus gastroenteritis patients; no. 10 to 18, nonpatients.

<sup>b</sup> Within 2 weeks after primary rotavirus infection, immune adherence hemagglutination (IAHA) Ab titers are lower than complement-fixing (CF) titers. Thereafter, the former exceeds the latter (9).

<sup>c</sup> NT, Not tested.

Next, we examined Ab dissociability after secondary rubella infection (Table 3). The two cases showed decreases in Ab dissociability. Case 2 had high HI Ab titers but low Ab dissociability, in contrast to primary-infection cases who had high HI titers and high Ab dissociability (Table 2).

DISCUSSION

For single-serum diagnosis of virus infections, the detection of IgM Ab by immunosorbent assays (ELISA and solid-phase radioimmunoassay) is used increasingly. IgM Ab persists in the serum for a short time after the infection; thus, its detection may have diagnostic value. However, this method has some drawbacks. When specific IgM Ab is detected, a recent virus infection is considered likely. However, when it is not detected, a recent infection cannot

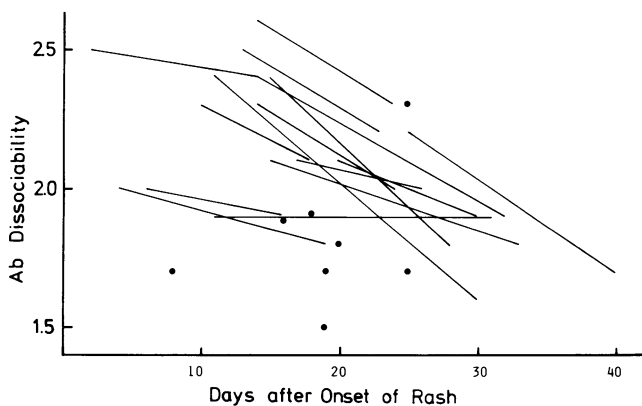


FIG. 2. Changes in Ab dissociability after primary rubella infection. Paired sera are connected by lines. Dots indicate single sera.

TABLE 2. Ab dissociability after primary rubella infection

Patient no.	Age (yr)	Sex	Days after onset of rash	HI titer	Ab dissociability
1	8	F	17	256	2.1
			26	128	2.0
2	31	F	14	1,024	2.6
			24	256	2.3
3	13	M	14	1,024	2.3
			25	512	2.0
4	19	M	11	512	1.9
			31	256	1.9
5	11	F	15	256	2.1
			33	256	1.8
6	10	F	2	32	2.5
			14	128	2.4
			32	128	1.9
7	33	M	11	128	2.4
			30	128	1.6
8	10	F	4	16	2.0
			19	256	1.8
9	7	F	10	512	2.3
			18	512	2.1
10	26	F	25	256	2.2
			40	128	1.7
11	26	F	15	256	2.4
			28	256	1.8
12	26	F	20	512	2.1
			30	512	1.9
13	26	M	6	1,024	2.0
			16	256	1.9
14	17	M	13	2,048	2.5
			23	2,048	2.2

completely be ruled out, since the extent of IgM Ab responses varies with each individual and some patients may not produce sufficient amounts of IgM Ab to be detected, although they respond with detectable IgG Ab. Also, this method may not always distinguish primary from secondary virus infections.

Another problem is false-positive results. Morgan-Capner et al. (18) found that some sera from infectious mononucleosis patients gave positive results for IgM Ab to unrelated rubella virus as detected by their IgM capture radioimmunoassay. Best et al. (2) reported that a commercially available kit for rubella IgM Ab also gave false-positive results in some sera. The heteroreactive results might be a problem more often in IgM than IgG assays. When the combining site of an Ab molecule has a fortuitous weak reactivity with an epitope of an unrelated virus antigen, IgM, which has ten combining sites, might attach more avidly than IgG to these antigens through multipoint binding (12).

Estimation of IgG Ab avidity may prove to be an alternative method to IgM Ab detection for single-serum diagnosis. Theoretically, this method would not be expected to have the potential for the false-negative and -positive results

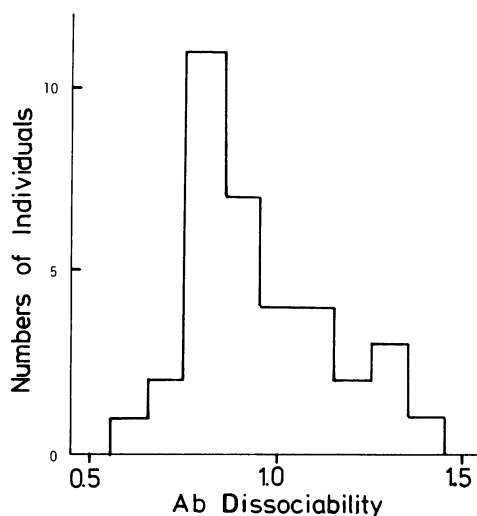


FIG. 3. Frequency in Ab dissociability among 35 rubella Ab-positive healthy individuals.

mentioned above. We have now devised a simple procedure for estimating relative Ab avidity and have shown the possibility of using this procedure for diagnostic purposes. It is interesting to note that in the few cases examined, this technique not only distinguished sera from a recent primary rubella infection from sera from a remote infection, but also distinguished sera from rubella reinfection (high HI titer and high avidity) from sera from a primary infection (high HI titer and low avidity) (Tables 2 and 3). General applicability of this procedure, however, awaits future evaluation.

Guanidine hydrochloride and chaotropic ions ( $\text{SCN}^-$ ,  $\text{I}^-$ ), which cause reversible denaturation of protein structure, are known to inhibit the formation of immune complexes (14) and have been exploited for elution of Abs or antigens in immunoaffinity chromatography (1, 4). Murphy et al. (19) found that stronger conditions for use of these reagents are needed for elution of Abs produced at late, as opposed to early, stages of immunization. We utilized guanidine for making stringent conditions for antigen-Ab reactions in the immunosorbent assay so that the binding of low-avidity Ab was inhibited, whereas high-avidity Ab was still allowed to bind to the antigens. The reagent probably affects the fine structure of both the antigenic determinant and the combin-

ing site of Ab, diminishing the association constant and thus preventing the loose-fitting Ab from holding to the antigens. For achieving such conditions, we had to use different concentrations of guanidine for different antigens: 0.5 M for JE and rubella viruses and 1.0 M for rotavirus. Rotavirus antigens are probably more stable against the denaturing effect of guanidine than are JE and rubella antigens. We propose calling the principle of this procedure stringent immunosorption.

It should be noted at this point that all the results described above were obtained by using freshly prepared antigens. Miyazawa et al. (17) reported that an ELISA kit for rubella IgG Ab (Rubelisa G; M. A. Bioproducts, Walkersville, Md.) gave low reactivity with sera taken within 1 month after the rash. We confirmed their observation and also found that isolated IgG fractions from the early sera gave low reactivity in the ELISA kit when their HI titers were fairly high, whereas IgG Ab of late sera had stronger reactivity in the ELISA but lower HI titers (S. Inouye and Y. Tsurukubo, unpublished data). We also saw a similar tendency with another kit (Rubazyme G; Abbott Laboratories, North Chicago, Ill.) and concluded that these kits are not suitable for our procedure, since the early sera already have low reactivity in the ELISA without addition of the denaturing reagent. We postulate that rubella virus antigens in these kits undergo a slight denaturation during freeze-drying and storage, and thus low-avidity Ab in early sera are no longer reactive with the denatured antigens even in the absence of guanidine. Therefore, we wish to emphasize that the character of antigen preparations is important for our procedure to give valid results.

Finally, this procedure might also be useful for analysis of antigenic relatedness between closely related viruses. Abs in a hyperimmune serum are considered to react less avidly with heterologous than with homologous viruses. It is possible that a heterologous reaction may be differentiated from a homologous one by the stringent immunosorption technique.

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TABLE 3. Ab dissociability after secondary rubella infection

Case no.	Date of bleeding	HI titer	Ab dissociability
1 <sup>a</sup>	3-30-76	8	1.1
	5-10-76	16	0.5
	5-21-76	16	0.5
2 <sup>b</sup>	4-13-81	16	0.9
	5-22-81	512	0.5
	9-19-81	512	0.5

<sup>a</sup> A 27-year-old female who had swelling of cervical lymph nodes after her child got rubella in April 1976.

<sup>b</sup> A 23-year-old female who had contact with a child with rubella in April 1981 but did not develop any symptoms of disease.



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