

Detection of Immunoglobulin G and A Antibodies to Rubella Virus in Urine and Antibody Responses to Vaccine-Induced Infection

SHIGEO TAKAHASHI, FUSAICHI MACHIKAWA, ATSUNARI NODA, TETSUYA ODA,
AND TETSUYA TACHIKAWA*

Department of Diagnostic Reagents Development, Otsuka Pharmaceutical Co., Ltd., Tokushima 771-01, Japan

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Urine and serum samples from 89 healthy volunteers and three healthy individuals who underwent rubella vaccination were tested for immunoglobulin G (IgG), IgA, and IgM to rubella virus (RV) by enzyme-linked immunosorbent assay methods. Subjects with positive ($n = 68$) or negative ($n = 21$) results for serum IgG were exactly the same as those with the corresponding results for urinary IgG. Both urinary and serum IgG levels remained elevated from the 3rd or 4th week after vaccination until the end of the study. Both urinary IgA and serum IgM levels tended to increase rapidly between the 3rd and 5th week and then gradually decrease until the end of the study, but the levels of both remained positive except for one sample each at the end (26th week). On the other hand, the ratio of anti-RV IgA titer to anti-RV IgG titer in urine (urinary anti-RV IgA/IgG ratio) increased rapidly between the 3rd and 4th week after vaccination and then rapidly returned to the ratio levels of the subjects positive for serum IgG from among the healthy volunteers. In summary, detection of urinary anti-RV IgG should be useful for screening for previous RV infection, and measurement of urinary anti-RV IgA/IgG ratio might be useful for diagnosing recent infection.

Infection by rubella virus (RV), the sole member of the genus *Rubivirus* of the family *Togaviridae*, is usually a self-limited disease that occurs mainly between infancy and puberty. Symptoms are usually mild and similar to those of other viral diseases with rash such as measles, scarlatina, infectious erythema, exanthema subitum, and infectious mononucleosis (17). Therefore, differential diagnosis of these is required, and blood samples are usually used for this purpose. However, it is difficult to collect blood samples from infants and young children. Although urine-based antibody tests have been developed for screening of infectious diseases (5, 10, 15), saliva-based antibody tests have been developed only for RV (13, 14). More importantly, the fetuses of women infected with RV during the first trimester of pregnancy have a high rate of congenital rubella syndrome (17). To reduce the risk of development of congenital rubella syndrome, a hemagglutination inhibition (HI) test has routinely been performed to detect anti-RV antibody in serum in pregnant women. This method is useful for screening for infection but is not useful for determining when viral infection occurred. Enzyme-linked immunosorbent assay (ELISA) for anti-RV immunoglobulin M (IgM) in serum is widely used for detection of recent infection. Anti-RV IgM has been reported to appear in serum from the 2nd or 3rd week after infection and to disappear by the end of the 1st or 2nd month (3, 4, 6–8, 16). Subsequently, however, it has also been demonstrated that IgM can be detected in serum for longer periods, up to a year after infection (1, 2, 11, 12). Elevation of HI titers for a period of 1 or 2 weeks has also been used for detection of recent infection; however, specimens must be collected at intervals of more than 1 week for the assay. In this study, we have for the first time developed an ELISA method for detection of anti-RV IgG and IgA antibodies in human urine and compared its usefulness with that of a

commercially available kit for serum antibody detection. We also examined the urinary anti-RV IgA/IgG ratio for detection of recent infection, using vaccinated subjects.

MATERIALS AND METHODS

Specimens. Random single-void urine and serum samples were collected at the same time from 89 healthy volunteers, including 83 males (21 to 51 years old; mean age, 36.2 years) and 6 females (21 to 37 years old; mean age, 27.3 years); five of the females had undergone rubella vaccination when they were 13 or 14 years old. In addition, three healthy males (31 or 32 years old) underwent one injection of rubella vaccine (TCRB19 strain; Chiba Serum Institute, Chiba, Japan), and random single-void urine and serum samples were collected from them before vaccination and at several points up to the 26th week after vaccination. Unfortunately, we failed to collect samples from one subject at the 4th week and from another subject at the 22nd week.

ELISA for anti-RV antibodies in urine. Wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μ l of a 1- μ g/ml RV antigen solution (Biodesign, Kennebunk, Maine) in Dulbecco's phosphate-buffered saline, pH 7.4 (PBS) (Nissui Pharmaceutical, Tokyo, Japan). After overnight incubation at 4°C, the unoccupied binding sites were blocked by incubation overnight at 4°C with Dulbecco's PBS containing 1% bovine serum albumin (Seikagaku Kogyo, Tokyo, Japan) and 5% sucrose (Wako Pure Chemicals, Osaka, Japan). The plates were air dried after aspiration of the blocking solution and stored at 4°C with desiccant until use. The plates were incubated with 20 μ l of 0.2 M Tris chloride buffer, pH 7.3, containing 0.14 M sodium chloride, 2% casein (Wako Pure Chemicals), 0.5% bovine serum albumin, 0.05% Tween 20, and 0.1% sodium azide and with 100- μ l urine samples at 37°C for 1 h. Plates were then rinsed with PBS containing 0.05% Tween 20 and 0.05% sodium azide (PBS-T) and incubated at 37°C for 1 h with either 100 μ l of horseradish peroxidase-conjugated anti-human IgG (Jackson, West Grove, Pa.) or horseradish peroxidase-conjugated anti-human IgA antibody (Jackson). The plates were then rinsed with PBS-T and incubated with 100 μ l of 3,3',5,5'-tetramethylbenzidine soluble solution (Scytek Laboratories, Logan, Utah) for color development. The reaction was stopped by adding 100 μ l of stop solution for 3,3',5,5'-tetramethylbenzidine (Scytek Laboratories). Optical density (OD) was measured at 450 nm by using a plate reader (Molecular Devices Vmax, Sunnyvale, Calif.). The mean value plus 3 standard deviations calculated from the test results for subjects who had negative results for serum IgG was used as a cutoff value for each urinary assay. The cutoff indices ($OD_{\text{sample}}/OD_{\text{cutoff}}$) were used for evaluation of each urinary assay, and the anti-RV IgA/IgG ratios were calculated by using the cutoff indices. The mean value plus 5 standard deviations calculated from the anti-RV IgA/IgG ratios of the healthy volunteers who had positive results for serum IgG was used as a cutoff value for evaluation of the urinary anti-RV IgA/IgG ratio.

* Corresponding author. Mailing address: Otsuka Pharmaceutical Co., 463-10 Kagasuno, Kawauchi, Tokushima 771-01, Japan. Phone: 81-886-65-6156. Fax: 81-886-65-8119. E-mail: tetsuyat@po.iijnet.or.jp.

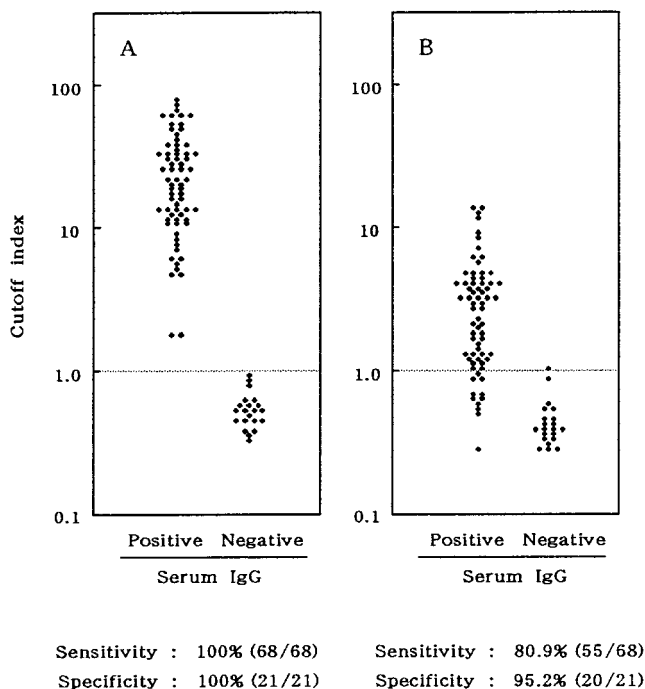


FIG. 1. Levels of urinary IgG and IgA antibody to RV in healthy volunteers. The division of subjects into positive and negative groups was based on serum IgG levels. Horizontal lines indicate the cutoff value. (A) Urinary IgG level; (B) urinary IgA level.

ELISA for anti-RV antibodies in serum. Anti-RV IgG and IgM antibodies in serum were measured by using commercially available ELISA kits (Denka Seiken, Tokyo, Japan) in accordance with the manufacturer's instructions.

RESULTS

Detection of urinary anti-RV IgG and IgA antibodies in healthy volunteers. Serum and urine samples were collected at the same time from 89 healthy volunteers, and levels of serum IgG, urinary IgG, and urinary IgA against RV were measured by the ELISA method developed in this study or the commercially available ELISA kit (serum IgG). When subjects were divided into a positive group ($n = 68$) and negative group ($n = 21$) based on the results of the serum IgG assay, both the urinary IgG and the urinary IgA levels were significantly higher ($P < 0.001$ [unpaired t test]) in the positive group than in the negative group (Fig. 1). Notably, both the sensitivity and the specificity of the urinary IgG assay were perfect (100%) compared to those for serum IgG, indicating that urine samples can be used as an alternative to serum samples for RV antibody screening. On the other hand, only 56 of 68 (82%) subjects in the serum IgG assay-positive group had positive results for urinary IgA. The urinary IgA assay was therefore not useful for screening.

Changes in urinary and serum anti-RV antibody levels after vaccination. Three healthy subjects underwent rubella vaccination, and their levels of urinary IgG and IgA antibody and serum IgG and IgM antibody against RV were measured by using samples collected before and at regular intervals after vaccination (Fig. 2). The serum and urinary IgG levels remained elevated from the 3rd or 4th week after vaccination. The serum IgM and urinary IgA levels rapidly increased between the 3rd and 5th week and then decreased gradually but remained positive for a long time, as shown in Fig. 2. Individual urinary antibody levels fluctuated during the test period due to

variability in the concentrations of urine samples. In addition, individual profiles of urinary IgG levels differed considerably from those of urinary IgA levels between the 3rd and 8th week, while these profiles were quite similar to each other after the 9th week. This type of relationship was not observed between serum IgG and IgM levels.

Changes in urinary anti-RV IgA/IgG ratios after vaccination. Anti-RV IgA/IgG ratios for the urine samples from the vaccinated subjects were calculated and compared with the ratios calculated by using subjects positive for serum IgG from among the healthy volunteers (Fig. 3). The ratios increased rapidly between the 3rd and 4th week after vaccination and then decreased rapidly to the levels found for the positive volunteers. All the ratios during the period from the 3rd to the 8th week were beyond the cutoff value except for one sample at the 7th week. None of the ratios for the samples collected after the 9th week or those for the samples from the positive volunteers who were assumed to have been infected or vaccinated with RV in the past were beyond the cutoff value.

DISCUSSION

Most rubella patients are infants or young children. However, it is difficult to collect blood samples from small children for detection of anti-RV antibody. The urine-based assay methods developed in this study should be helpful in solving this problem, since urine samples were demonstrated to be a useful alternative to serum samples for detection of anti-RV IgG antibody. Urinary anti-RV IgA antibody was also detected in many samples, but the sensitivity was lower than those for serum IgG and urinary IgG antibodies. However, urinary IgA antibody levels as well as serum IgM antibody levels increased earlier than urinary IgG and serum IgG after rubella vaccination. High urinary IgA levels were maintained for a long time as observed for serum IgA (2, 9, 11), and the individual urinary IgA levels fluctuated markedly during the test period due to variability in urine concentration. Therefore, urinary IgA might not be a useful marker for detection of recent RV infection. Although serum IgM is a marker of recent infection, positive levels of serum IgM were maintained for a long time after vaccination, as shown in Fig. 2. This observation has been reported not only for vaccine-induced infection but also for natural infection (1, 2, 11, 12). It is also recommended that an increase in the HI test titer in 1 or 2 weeks should be tested when recent infection is suspected.

On the other hand, urinary anti-RV IgA/IgG ratios dramatically increased and then decreased, with a peak at the 4th week in the primary period after vaccination, even though urinary IgA and IgG levels remained positive at least until the 26th week after vaccination in most cases. This was caused by the gradual increase in urinary IgG level and rapid increase in urinary IgA level as a primary antibody response. Ratios did not fluctuate markedly because the effects of variability in concentration in urine must have been eliminated by division of IgA level by IgG level for the same urine sample in the calculation of ratios.

Serum anti-RV IgM/IgG ratio was not a better indicator of recent infection than urinary IgA/IgG ratio (data not shown). Although serum IgG ELISA is usually performed as a direct assay using antigen-coated microplates, serum IgM ELISA requires absorption of IgG in serum samples as pretreatment or a capture assay method using anti-human IgM antibody-coated plates. Poorer indication of recent infection by serum IgM/IgG ratio may be due to differences in assay methods. On the other hand, the ELISAs developed for urinary IgG and IgA in this study are direct assays using antigen-coated microplates

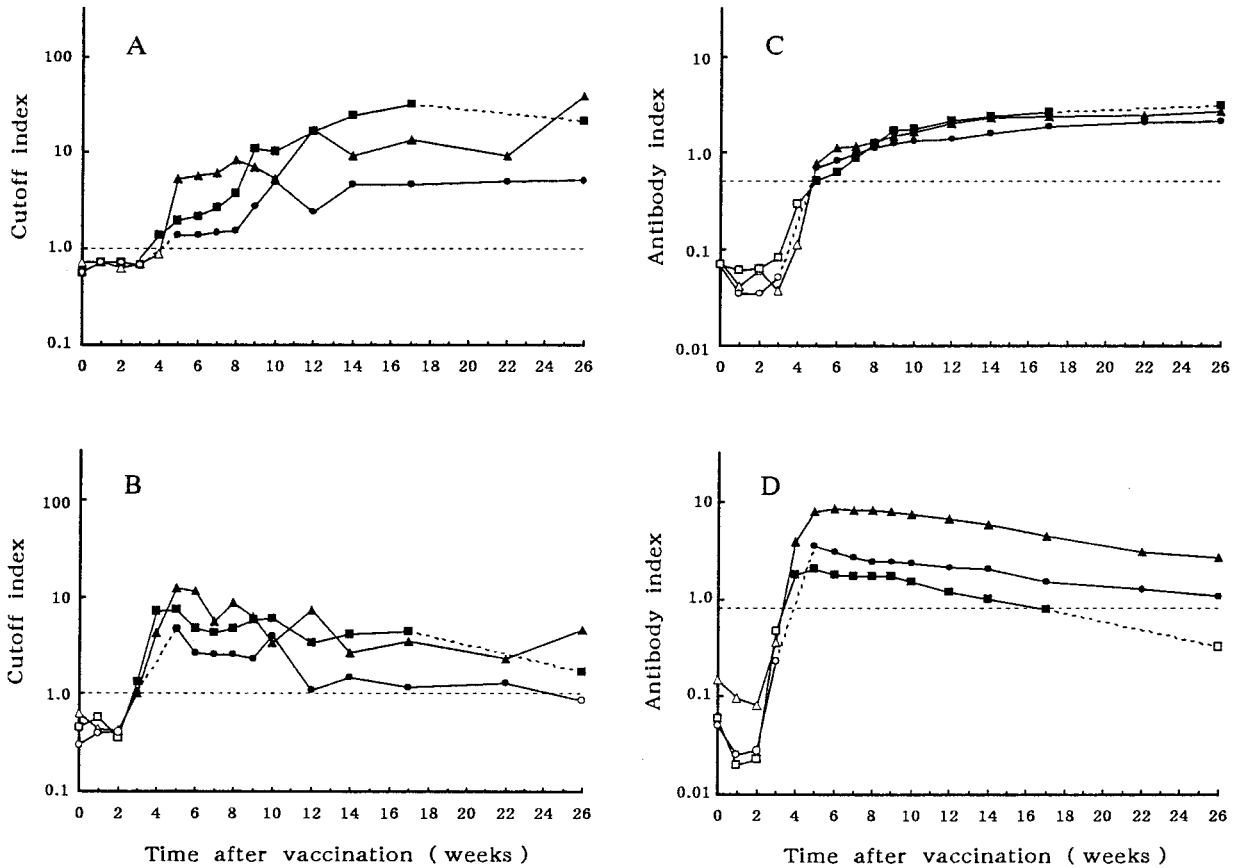


FIG. 2. Changes over time in anti-RV antibody levels in urine and serum after vaccination. Changes over time in titers of urinary IgG (A), urinary IgA (B), serum IgG (C), and serum IgM (D) against RV are indicated. ●, positive sample; ○, negative sample. Samples with indeterminate values in serum assays were classified as positive. Dashed portions of the curves indicate failure to collect a sample. Horizontal lines indicate cutoff values. Circles, squares, and triangles correspond to three subjects.

without any pretreatment including sample dilution and require common materials and procedures except for enzyme conjugates (anti-human IgG or anti-human IgA). The common

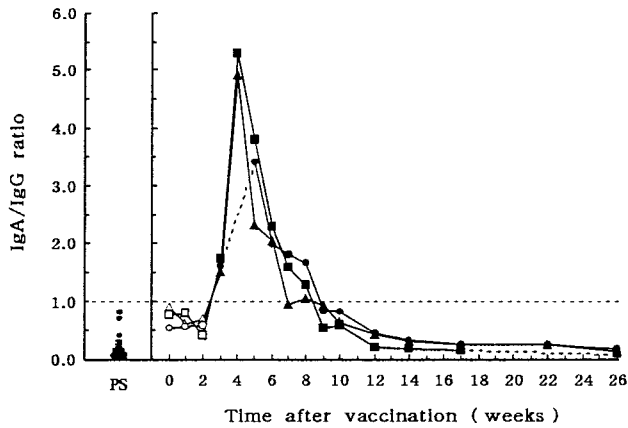


FIG. 3. Changes over time in ratios of the titer of IgA antibody to RV in urine after vaccination, compared with the ratios for IgG-positive subjects (PS) from among the healthy volunteers. Closed symbols, samples positive for urinary anti-RV IgG or IgA; open symbols, samples negative for urinary anti-RV IgG and IgA. Dashed portions of the curves indicate failure to collect a sample. The horizontal line indicates the cutoff value of the ratio. Circles, squares, and triangles correspond to three subjects.

assay method may demonstrate the usefulness of the urinary IgA/IgG ratio for detection of recent infection.

In conclusion, we have developed for the first time ELISA methods for detection of urinary IgG and IgA antibodies to RV. The assay methods devised are simple and do not require any pretreatment of samples such as absorption of IgG or dilution of samples prior to setup. Random single-void urine samples are extremely easy to collect from infants and young children. Our findings suggest that direct testing of urine samples for RV-specific IgG provides an efficient alternative to serum-based assays. In addition, measurement of the ratio of IgA titer to IgG titer in the same urine sample may permit more accurate diagnosis of recent RV infection sooner after infection than serum-based IgM assays. Therefore, our urine-based assays should be useful for screening for RV-specific antibody, and they may also be helpful in diagnosing recent infection with RV. To confirm the usefulness of our assays, further examination using samples from patients who were recently and naturally infected with RV will be necessary.

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