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Subclass Distribution of Rubella Virus-Specific Immunoglobulin G

G. ANNIKA LINDE

Department of Virology, National Bacteriological Laboratory, and Department of Virology, Karolinska Institute, S-105 21 Stockholm, Sweden

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An enzyme-linked immunosorbent assay was used to study the subclass distribution of rubella virus-specific immunoglobulin G (IgG) in 97 serum samples from healthy donors and from patients with recent or remote rubella infections. Plastic beads coated with rubella antigen were incubated with test serum and then with monoclonal antibodies to the four human subclasses of IgG. Rubella virus-specific IgG1 was present in all serum samples containing rubella virus-specific IgG antibodies. Rubella virus-specific IgG2 was present in 1 of 35 samples from healthy donors that also contained specific IgG1. Rubella virus-specific IgG3 was found in serum samples from patients with recent rubella infections but had disappeared by 6 months after the onset of symptoms. Rubella virus-specific IgG4 was found in low amounts in 7 of 35 samples from healthy immune donors. Of 20 serum samples that were negative by other serological techniques, 8 gave absorbances above cutoff levels in the assays for rubella virus-specific total IgG and IgG1. In 1 of 20 serum samples, the assays for total IgG and IgG2 were positive. High absorbance in the assay for rubella virus-specific IgG4 was found in one serum. This serum was negative in all other assays for rubella virus-specific antibodies.

The four subclasses of human immunoglobulin G (IgG) respond differently to various types of antigens. IgG2 seems to be the major reactant to bacterial glycoproteins (8, 16). For virus-specific IgG, subclass distribution to some of the herpesviruses (9, 17, 20, 24), hepatitis B virus (13), and rubella virus in serum samples from healthy donors (17, 18) has been studied in immunoassays with subclass-specific monoclonal antibodies (mabs). IgG1, IgG3, and sometimes IgG4 are the IgG antibodies that have been shown to be produced in response to these viruses.

Rubella infection normally is an uncomplicated disease, but chronic rubella-associated arthritis and encephalitis may occur (2, 6, 7, 22, 28). The level of rubella antibody is often normal in rubella-associated arthritis (2). A serological method that indicates antigenic stimulation in cases of chronic infections would be very valuable. When low levels of rubella virus-specific antibodies are present before vaccination, complications seem to be more frequent than in seronegative individuals (21). Many samples seem to contain low amounts of rubella antibodies by newer sensitive assays although conventional serology is negative (1, 21, 25). Rubella reinfection seldom causes fetal damage (3). To distinguish primary rubella from reinfection is sometimes difficult; therefore, more reliable methods are also desirable for this differentiation.

We describe a simple method for learning more about the IgG response to rubella virus in healthy and diseased subjects. Using monoclonal antibodies to the human IgG subclasses with rubella antigen bound to a solid phase, we have developed an enzyme-linked immunosorbent assay (ELISA) to measure rubella virus-specific IgG subclasses. With this method we have found differences in rubella virus-specific subclass reactivity between healthy and rubella virus-infected individuals which suggest that subclass analyses might improve the capacity to detect low amounts of rubella virus-specific antibodies. We have also found differences in subclass distribution between the latent viruses previously examined (9, 20) and rubella virus without known latency. The reagents used are commercially available, which facilitates further studies for research or clinical purposes.

MATERIALS AND METHODS

Rubella virus IgG. (i) Total rubella virus-specific IgG. To estimate the total amount of rubella virus-specific IgG in serum samples, the Rubazyme G kit (Abbott Laboratories, North Chicago, Ill.) with the buffers and reactants provided was used entirely by the instructions of the manufacturer. Plastic beads coated with rubella virus were incubated with 10 μl of serum diluted in 200 μl (dilution, 1/21) of incubation buffer. After rinsing, 200 μl of peroxidase-labeled goat anti-human IgG was added to the beads, and finally they were incubated with 300 μl of orthophenyldiamine substrate solution. After 30 min at room temperature, the reaction was stopped with 1 N hydrochloric acid. A_{492} was read in a Quantum spectrophotometer (Abbott).

(ii) Assays for rubella virus-specific IgG subclasses. Four rubella antigen-coated beads were incubated with serum diluted 1/21 as described for total IgG. After rinsing the beads, we added 200 μl of immune ascitic fluid from mice, diluted in the incubation buffer (Abbott). The ascitic fluid contained mabs (Seward Laboratories, London, England). IgG1 (clone BAM 15) was diluted to 1/2,000, IgG2 (clone BAM 14) was diluted to 1/200, IgG3 (clone BAM 8) was diluted to 1/2,000, and IgG4 (clone BAM 11) was diluted to 1/800. The titration procedure for determining optimal dilutions of mabs has been described (9). When the mabs were added, a fifth bead was incubated with the serum of the patient for estimation of total rubella virus-specific IgG (see above). The conjugate provided in the Rubazyme kit was added to this bead. To the beads used for subclass analyses, we added 200 μl of peroxidase-labeled rabbit anti-mouse immunoglobulin (Dakopatts A/S, Copenhagen, Denmark), absorbed and diluted as previously described (9). Substrate reaction and reading of A_{492} were performed as described for total IgG. All incubations were for 1 h in a water bath at 37°C unless otherwise noted.

(iii) Analyses of results. The Rubazyme G kit contains one rubella antibody-negative, one high positive, and one low positive control serum. As described in the Rubazyme manual, the mean A_{492} for total rubella virus-specific IgG in a serum examined in duplicate is divided by the mean A_{492} for the low positive serum. If this ratio (Rubazyme index) is >1, the patient should be regarded as immune to rubella. If the index is <1, the patient has no rubella antibodies or insufficient antibody to provide protection against rubella infection. To evaluate the subclass reactivities, the negative control serum was examined for total IgG and for IgG subclasses. The mean A_{492} (plus three standard deviations) for 10 examinations (Table 1) was taken as the preliminary background level.

Other examinations for rubella virus-specific antibodies. (i) HAI assay. The hemagglutination inhibition assay (HAI) test (15) is used routinely at our laboratory. Rubella hemagglutinin antigen is purchased from Orion, Helsinki, Finland. Serum samples are treated with manganese-chloride-heparin to eliminate nonspecific inhibitors. Human rhesus-negative group O erythrocytes are used as the indicator system. Serum samples are diluted twofold, starting from a 1/8 dilution. Full or partial agglutination inhibition at a serum dilution of 1/8 indicates the presence of antibodies but questionable immunity to rubella. Complete inhibition at a dilution of 1/16 indicates full immunity. The HAI titer of the Rubazyme low positive control was 16.

(ii) HIG test. The hemolysis-in-gel test (HIG) (19) is also routinely performed. Pigeon erythrocytes coated with hemagglutinin antigen (Orion) are used. Undiluted serum (10 μl) is applied in wells 4 mm in width. A hemolytic zone of <8 mm in the gel indicates questionable rubella immunity. A zone of ≥1 mm is required for immunity. The Rubazyme low positive control gave a HIG zone of 8 mm.

TABLE 1. Mean A_{492} and standard deviation of 10 examinations for total IgG and IgG1 to 4 for the negative, low positive, and positive controls provided in the Rubazyme kit and for nine healthy individuals with Rubazyme indexes of <1 and absorbances for total IgG of <0.2

Serum (type of IgG)	Mean A_{492} (±SD)	Background A_{492}[a]
Negative control		
Total IgG	0.08 (0.04)	0.2
IgG1	0.16 (0.05)	0.3
IgG2	0.19 (0.07)	0.4
IgG3	0.18 (0.04)	0.3
IgG4	0.09 (0.03)	0.2
Low positive control		
Total IgG	0.34 (0.17)	
IgG1	0.88 (0.20)	
IgG2	0.17 (0.08)	
IgG3	0.17 (0.06)	
IgG4	0.11 (0.04)	
High positive control		
Total IgG	1.14 (0.45)	
IgG1	1.70 (0.30)	
IgG2	0.21 (0.08)	
IgG3	0.21 (0.05)	
IgG4	0.13 (0.04)	
Nine negative serum samples		
Total IgG	0.09 (0.04)	0.2
IgG1	0.16 (0.16)	0.3
IgG2	0.23 (0.04)	0.4
IgG3	0.17 (0.04)	0.3
IgG4	0.16 (0.05)	0.3

[a] Mean A_{492} plus three standard deviations.

Tests for rubella virus-specific IgM. (i) HAI after sucrose density gradient fractionation. At least one serum from each patient with rubella infection was examined by HAI testing of the IgM fraction after sucrose density fractionation (26).

(ii) Rubazyme M test. For evaluation of IgM levels, Rubazyme M (Abbott) was used entirely by the directions of the manufacturer. The A_{492} of the low positive control provided for IgM was regarded as the lowest positive reaction. The samples used in this study were negative by latex agglutination for rheumatoid factor as measured by the RA-Test reagent kit (Hyland Laboratories Diagnostic Div., Malvern, Pa.).

(iii) Solid-phase reverse immunosorbent test. The solid-phase immunosorbent test (4) was used for eight serum samples to verify absence of rubella virus-specific IgM in two samples from a patient with seroconversion to rubella and in six samples from persons with high levels of rubella virus IgG antibodies.

Serum samples. A total of 97 serum samples were examined. All had been stored at −20°C and frozen and thawed at least once before the study began.

Samples from persons evaluated as nonimmune to rubella. Serum samples from 20 individuals (age range, newborn to 41 years) who had been examined with HAI, HIG, and Rubazyme G tests in 1982 all had a Rubazyme index of <1. To find out whether the use of subclass analyses would increase detection of low amounts of rubella antibodies, we chose samples with indexes ranging from 0.13 to 0.99. The assumption was that a high index could be a sign of low amounts of specific antibodies. HIG zones and HAI titers for five of the serum samples that were weakly positive in these assays are given in Table 2. The specimens that were not drawn from healthy, unvaccinated donors are also indicated in Table 2. The samples had been sent to the laboratory for determination of rubella immunity and, in one case, because of congenital malformations.

Serum samples from healthy individuals evaluated as immune to rubella. Serum samples from each of 35 healthy individuals (age range, newborn to 52 years), with Rubazyme indexes of >1, HAI titers of >16, and HIG zones of >8 mm, were examined. Of the 35 samples, 15 were from healthy donors, and 20 had been submitted to the laboratory for determination of rubella immunity.

Serum samples from patients with current rubella. A total of 52 serum samples were from 19 patients (age range, 5 to 46 years) with clinical, uncomplicated rubella infection. The diagnosis was proven by seroconversion or a significant rise in titer to rubella in the HAI test and by a positive rubella HAI of the IgM fraction in at least one serum from each patient. The HAI-negative acute samples from two of the patients could not be used in the study due to lack of material.

For presentation of the results, the samples were divided into five groups after all examinations had been performed. Samples (10) drawn on days 0 to 3 after the onset of rubella symptoms were evaluated as acute samples. Samples (10) drawn on days 4 to 14 of the disease were taken in early convalescence. Samples (12) taken on days 15 to 60 after disease were late convalescent-phase serum samples. Three of these were drawn at day 60; the rest were drawn before day 30. Eight patients were bled on day 90 and on day 180 after the disease. They formed two follow-up groups.

TABLE 2. HAI titer, HIG zone, Rubazyme index, and A_{492} for total IgG and IgG subclasses for 11 serum samples with Rubazyme indexes of <1 and A_{492} above the background in subclass assays

Patient no.	HAI titer	HIG zone (mm)	Rubazyme index	A_{492}				
				Total IgG	IgG1	IgG2	IgG3	IgG4
1[a]	≤8	6	0.96	0.50	0.83	<0.4	<0.3	<0.3
2[a]	<8	<4	0.61	0.32	0.55	<0.4	<0.3	<0.3
3[b]	<8	7	0.92	0.48	0.62	<0.4	<0.3	<0.3
4	≤8	<4	0.89	0.47	0.58	<0.4	<0.3	<0.3
5	≤8	<4	0.82	0.43	0.63	<0.4	<0.3	<0.3
6[c]	≤8	<4	0.80	0.41	0.52	<0.4	<0.3	<0.3
7	<8	<4	0.74	0.38	0.3	0.71	<0.3	<0.3
8	<8	<4	0.66	0.34	0.52	<0.4	<0.3	<0.3
9	<8	<4	0.55	0.27	0.42	<0.4	<0.3	<0.3
10	<8	<4	0.24	0.14	0.3	<0.4	<0.3	1.08
11[d]	≤8–16	<4	0.82	0.42	0.75	<0.4	0.39	<0.3

[a] Patients 1 and 2 were a newborn infant with malformations and the mother of the infant, respectively.
[b] Vaccinated in 1975.
[c] Vaccinated in 1978.
[d] Rubella virus IgM positive.

The samples in the first three groups were submitted to the department for routine serology. The follow-up samples were collected for this study. Four samples from one patient with no detectable specific IgM but with seroconversion in tests for rubella virus-specific IgG are discussed separately.

RESULTS

Background levels in assays for total rubella virus-specific IgG and IgG subclasses. Mean A_{492} ranges and standard deviations for 10 examinations of total rubella virus-specific IgG and IgG subclasses for the negative control provided with the Rubazyme G kit are presented in Table 1. The mean absorbance plus three standard deviations for the negative control was regarded as a preliminary background level in the following assays.

Serum samples with a Rubazyme index of <1. We reexamined samples from 20 patients with indexes of <1 in 1982. Of these 20, 9 had A_{492}s below the preliminary background levels in all assays (Table 1) and were considered to lack rubella antibodies. The Rubazyme index was below 0.45 for these samples. The mean A_{492} and standard deviation in the IgG4 assay were slightly higher for those nine serum samples than for the negative control. The total amount of IgG4 in serum varies substantially in different individuals (12). This variation might explain the somewhat higher variability in the IgG4 amounts assayed in samples from different individuals. The background level for IgG4 was therefore raised to an A_{492} of 0.3. The background levels for the other assays were the same as that when the negative serum provided in the kit was examined.

The results for the remaining 11 specimens with a Rubazyme index <1 are presented in Table 2. When the A_{492} for IgG1 or IgG2 was above cutoff (samples 1 through 9), the A_{492} for total IgG also exceeded the established background level. Sample 10 (Table 2) gave the highest A_{492} for IgG4 found in the whole study. The A_{492} for total IgG was not above 0.2 for this sample. One serum contained rubella virus-specific IgG3, which made us suspect a current rubella infection in the donor. This was verified by the clinician and by positive IgM assays (HAI after gradient separation, solid-phase reverse immunosorbent test, and Rubazyme M).

In this study, the main aim was a comparison between ELISA results for total and subclass IgG. That weakly positive reactions always indicate past rubella infection was not verified. The absorbances for samples 8 through 10 (Table 2) might be due to nonspecific reactions. In the case of the sera from the mother and the newborn child (Table 2, patients 1 and 2), however, the low A_{492} in the serum from the mother seems to be specific, as a mother who lacks rubella virus antibodies cannot give birth to a rubella virus antibody-positive child.

Healthy individuals with Rubazyme ratios >1. Serum samples from 35 healthy individuals had a positive Rubazyme index and positive HAI and HIG results. The results of analyses for rubella virus-specific subclasses are presented in Fig. 1. IgG1 was the predominant antibody. Traces of IgG2 were found in one serum, and traces of IgG4 were found in seven samples.

Patients with current rubella infection. The A_{492}s for rubella virus-specific IgM, total IgG, and IgG subclasses for 48 samples from 18 patients with current, primary rubella infections are presented in Fig. 2 and 3.

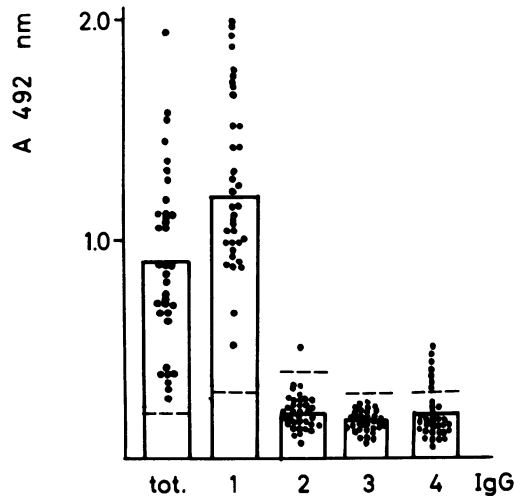

FIG. 1. A_{492} for rubella virus-specific total IgG and IgG subclasses in 35 serum samples from healthy donors with Rubazyme indexes of >1. ——, Mean A_{492}; – – –, background A_{492}. tot, Total IgG.

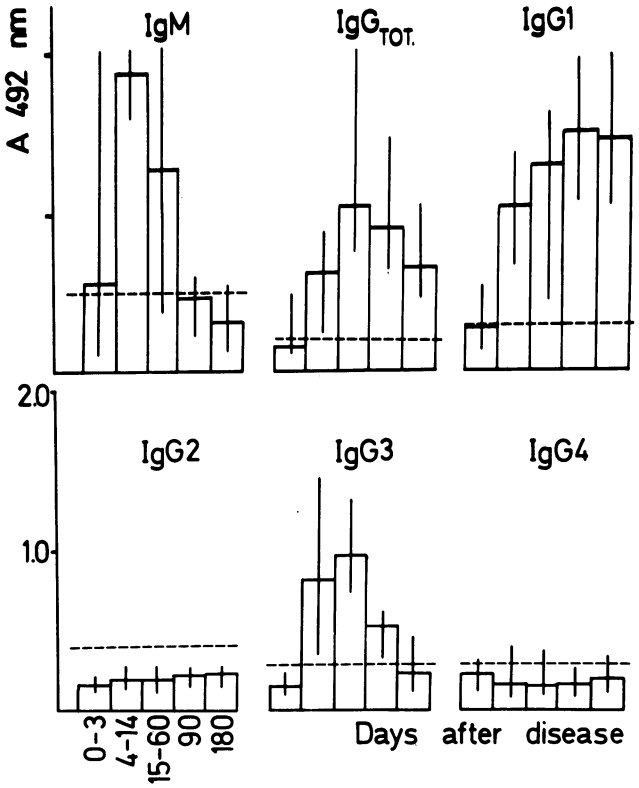

FIG. 2 Development of mean and range for the A_{492}s of rubella virus-specific IgM, total IgG, and IgG subclasses in 48 serum samples from 18 patients with current rubella infection. The numbers of samples (at days after onset of symptoms) were as follows: 10 (days 0 to 3), 10 (days 4 to 14), 12 (days 15 to 60), 8 (day 90), and 8 (day 180). – – –, Background A_{490}.

Samples drawn from days 0 to 3 after the onset of infection often contained low amounts of rubella virus-specific IgM or IgG1. Two samples lacked rubella antibodies of all kinds. From day 4, all samples were positive in the assays for IgM, total IgG, IgG1, and IgG3. The rubella virus-specific IgG3 reached its peak in samples drawn from days 14 through 60 after onset of the disease and had disappeared from seven of eight samples drawn 6 months after onset of the disease. In three samples drawn at day 60, the absorbance for rubella virus-specific IgM had fallen considerably, whereas the absorbance for IgG3 remained high.

We examined four serum samples from one patient with an IgM-negative rubella infection. A serum drawn 2 years before infection gave an A_{492} for total IgG of 0.4 but was otherwise negative. In samples from early and late convalescence, this patient had rubella virus-specific total IgG and IgG1. The A_{492} for IgG3 was just above the background level in a serum sample drawn 2 years after onset of the disease. The A_{492} for total rubella IgG and IgG1 had risen further, but the IgG3 had disappeared. We suspect that this patient had a rubella reinfection, A_{492} for total IgG being the only sign of her past rubella. Unfortunately, we had no more samples from such patients.

DISCUSSION

IgG1 appears to be the predominant rubella IgG subclass seen in healthy and rubella virus-infected individuals. This has also been found in other recent studies (17, 18). We found rubella virus-specific IgG3 antibodies only in connection with recent infection. Rubella virus-specific IgG4 was variable, in keeping with IgG4 for other viral antigens examined (9, 13, 17, 20). The use of background A_{492} levels appears to reveal more information on low amounts of rubella virus-specific antibodies than does the use of a Rubazyme index. This is not surprising, as the Rubazyme low positive control contains measurable HAI antibodies. In this work we did not show that the use of background A_{492} ensures that the reactivity is specific, but ELISA examinations for rubella antibodies have previously been reported to be more sensitive than the HAI test (1, 21, 25). It has also been suggested that an index of 0.8 indicates presence of rubella virus-specific antibodies (5). In this study the important result was that the ELISA for total IgG revealed the antibodies that were found in the assays for IgG1 and IgG2 but did not detect the IgG4. The frequency and significance of virus-specific IgG4 need to be further examined. If IgG4 is proven to be a sign of past rubella, it must be verified that ELISA systems for total IgG also measure this subclass.

The validity of our findings depends upon the sensitivity and specificity of the reactants used. We have previously compared the Rubazyme index with results in HIG tests for 700 serum samples for clinical purposes. The agreement regarding immunity between HIG zones of >8 mm and Rubazyme indexes of >1 was 99%. Data on the reliability of the Rubazyme test have also been published (14). We therefore regard the specificity and purity of the antigen to be well documented, although we cannot entirely exclude some nonspecific reactions since control antigen is not available. It is possible, also, that subclass reactivity towards rubella antigens produced in other ways than the Rubazyme antigen may differ from what we have found in this study.

The subclass specificity of the mabs has been analyzed previously and seems satisfactory (10, 23). The clone specific for IgG2 is not as efficient as the others. In the same ELISA system as we have used, however, the major response to bacterial antigens, and to malaria in some cases, has been shown to be of the IgG2 subclass (8, 27). We thus believe that IgG2 is not normally a major antiviral subclass.

The above background A_{492}s for rubella virus-specific IgG1 were higher than for total IgG (Fig. 1 and 2). The use of a third antibody in the subclass ELISAs probably elevates both specific and nonspecific absorbances. Further, the efficacy of conjugates may vary. We found a decrease in A_{492} for rubella virus-specific total IgG in parallel with the decrease of IgG3 in serum samples from patients with the disease. The goat anti-human IgG antiserum used in the Rubazyme test thus might react better with IgG3 than with

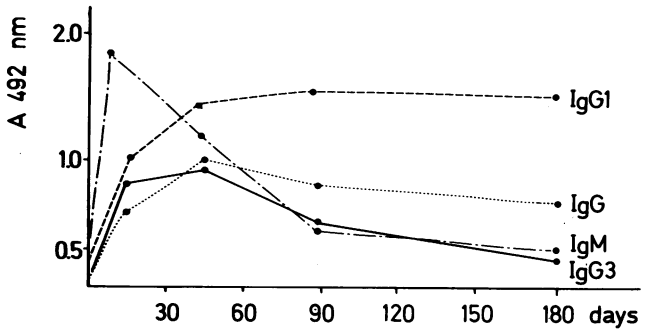

FIG. 3. The same mean A_{492}s as in Fig. 2 presented as curves. This shows the immunoglobulin kinetics.

IgG1. The IgG4 antibodies measured in one patient with no sign of rubella virus-specific total IgG indicate that the IgG conjugate has a low affinity for IgG4.

The most interesting finding in this study was the rise and fall of rubella virus-specific IgG3 in connection with rubella infection. The rise began after the production of IgM had started; the fall occurred shortly after the decrease of IgM. An interesting possibility is that a switch occurs in the heavy chain production from IgM to IgG3 (11). In one patient with rubella virus IgM-negative infection, the IgG3 was very low. This would support the idea that production of IgG3 is dependent on preceding IgM production in rubella infection. In herpesvirus infections virus-specific IgG3 is frequently found in healthy individuals (9, 20). Since those infections are latent, there is a continuous antigenic stimulation. IgG3-producing B-cells might have to be kept active by antigenic stimulation. A study of the levels of specific IgG3 in chronic infections caused by virus without latency would be of interest. IgG1-producing cells apparently have an immunological memory since specific IgG1 is the predominant antibody in healthy rubella-immune donors.

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