Sensitive Neutralization Test for Rubella Antibody

HIROSHI SATO,[†] PAUL ALBRECHT,^{1*} SAUL KRUGMAN,² AND FRANCIS A. ENNIS¹

*Food and Drug Administration, Bureau of Biologics, Division of Virology, Bethesda, Maryland 20014,¹ and
Department of Pediatrics, New York University School of Medicine, New York, New York 10016²*

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A modified rubella virus plaque neutralization test for measuring rubella antibody was developed based on the potentiation of the virus-antibody complex by heterologous anti-immunoglobulin. The test is highly sensitive, yielding titers on the average 50 to 100 times higher than the hemagglutination inhibition test or the conventional plaque neutralization test. The sensitivity of this enhanced neutralization test is somewhat limited by the existence of a prozone phenomenon which precludes testing of low-titered sera below a dilution of 1:16. No prozone effect was observed with cerebrospinal fluids. The specificity of the enhanced neutralization test was determined by seroconversion of individuals receiving rubella vaccine. Although the rubella hemagglutination inhibition test remains the test of choice in routine diagnostic and surveillance work, the enhanced rubella neutralization test is particularly useful in monitoring low-level antibody in the cerebrospinal fluid in patients with neurological disorders and in certain instances of vaccine failure.

Since the isolation of rubella virus in 1962 a number of tests for measuring rubella antibody have been developed (3, 4, 6, 11, 17, 18, 20; J. W. Safford, Jr., and R. Whittington, *Fed. Proc.* **35**: 813, 1976). The most widely adopted test for both clinical use and epidemiological surveillance is the hemagglutination inhibition (HI) test. The virus neutralization (Nt) test proved to be less sensitive than the HI test, and is cumbersome because of the difficulty in plaquing rubella virus in cell cultures. Recently we found that the Cendehill strain of rubella virus forms small, distinct, and regularly reproducible plaques in Vero cell cultures and that the test could readily be adapted to a semi-micro titration of virus infectivity for a neutralizing antibody assay (15; *Jpn. J. Med. Sci. Biol.*, in press). We further found that heterologous immunoglobulin directed against human immunoglobulin G (IgG) increased specific antibody titers to mumps virus by two orders of magnitude (15).

The present communication reports the development of a highly sensitive plaque Nt test for rubella antibody and its potential for monitoring vaccine performance and for studies of rubella infection of the central nervous system (CNS).

MATERIALS AND METHODS

Cell cultures and media. Vero cells at passage level 170 through 220 were supplied by the Tissue

Culture Section of the Division of Virology, Bureau of Biologics, Food and Drug Administration. Cells were grown in Eagle minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum. The serum was reduced to 5% in the maintenance medium.

Vero cell monolayers were grown on disposable plastic trays (Costar, Cambridge, Mass., or Linbro, Hamden, Conn.) containing 24 wells with a 16-mm diameter. The cell suspensions were seeded at 2.5×10^5 cells per ml, 1 ml per well. After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, the monolayers were confluent and ready to use.

Viruses. The attenuated Cendehill strain of rubella virus (Cendevax, Recherche et Industrie Therapeutiques, Genval, Belgium) (13) was used for the plaque Nt test. The vaccine strain was passaged five additional times in Vero cell cultures, which increased its titer from 5×10^4 to 10^7 plaque-forming units per ml.

The non-attenuated strain M-33 of rubella virus (10, 12) was received from Paul D. Parkman, Bureau of Biologics, Food and Drug Administration, in its third passage in BS-C-1 cells. Its titer was 10^4 tissue culture infective doses per 0.1 ml when tested in primary African green monkey kidney cell tube cultures.

Sera. Pre- and postimmunization sera were obtained from children immunized with monovalent measles, mumps, or rubella vaccines or with trivalent measles-mumps-rubella vaccine. In addition, rubella antibodies were determined in sera from young adults and elderly people whose preimmunization samples were obtained in the course of an influenza immunization study. The age of the different study groups, type of vaccine, and immunization interval, where applicable, are indicated in the results and/or in the respective tables.

AIG. The IgG fraction of rabbit serum against

[†] Present address: Department of Measles Virus, National Institute of Health of Japan, Tokyo, Japan.

human IgG (heavy and light chains) (anti-immunoglobulin [AIG]) was purchased from Cappel Laboratories (Cochranville, Pa.). It was used both with human and rhesus monkey immune serum, considering the broad cross-reactivity between human and rhesus monkey IgG. The concentration of specific antibody as given by the manufacturer was 2.5 to 2.8 mg/ml. The specific activity of the AIG preparation was determined in our laboratory by Ouchterlony double-diffusion test in plates using 0.35% agarose in phosphate-buffered saline (pH 7.2). Precipitation lines developed after 24 h of incubation with AIG diluted up to 1:32 when human IgG (1 mg/ml) was used as the antigen. The dilutions of AIG used in individual tests are given in the results.

Virus plaque Nt test. Fourfold serum dilutions were mixed with an equal volume (80 μ l) of rubella virus diluted so as to yield 20 to 25 plaque-forming units per well. After 1 h of incubation of 37°C, 80 μ l of tissue culture medium (conventional Nt test) or AIG (enhanced Nt test) was added to the serum-virus mixture, and incubation was continued for another 10 min at room temperature. A 0.1-ml aliquot was inoculated into each of two 16-mm wells on the plastic tray. After adsorption for 1 h at 37°C, the inoculum was replaced with 0.5 ml of overlay medium containing 0.5% agarose (Seakem, Marine Colloids Inc., Rockland, Maine) in maintenance medium. After incubation for 3 days at 35°C in a 5% CO₂ atmosphere, each well received another 0.5 ml of overlay with a 1:15,000 dilution of neutral red. After a total incubation of 7 days, the overlay was stripped, and the cell sheets were air dried.

The Nt antibody titer was defined as the reciprocal of serum or cerebrospinal fluid (CSF) dilution reducing the number of viral plaques per well by 50%. The dilution was calculated according to Kärber's method (7). Rubella human reference serum lot no. 1 (Bureau

of Biologics, Food and Drug Administration) was used as a reference in each test.

HI test. The HI test for rubella antibody was performed by the method of Stewart et al. (18) with slight modifications. Nonspecific virus inhibitors of rubella virus and natural hemagglutinins were removed by treatment with 25% kaolin followed by absorption with packed erythrocytes in Dulbecco phosphate-buffered saline (pH 7.4). The treatment with kaolin was omitted on CSF samples. The test was done in U-type microplates using 0.25% 1-day-old chicken erythrocytes and Veronal-buffered saline (pH 7.0), including 0.1% bovine serum albumin fraction V and 0.01% gelatin.

Experimental infection of rhesus monkeys. Rhesus monkeys (*Macaca mulatta*), weighing approximately 6 to 8 kg, were infected intravenously with 1.0 ml of strain M-33 of rubella virus.

At weekly intervals, serum and CSF were obtained and rubella antibody titers were determined. Throat swabs were taken, and virus isolation was attempted in primary African green monkey kidney tube cultures according to the interference method of Parkman et al. (12). The virus isolates were identified by neutralization with reference serum.

RESULTS

Determination of rubella antibody by the enhanced Nt test. Sera from 12 children immunized with rubella vaccine were tested by the HI test and by the conventional and the enhanced Nt tests. The conventional Nt test was in most instances less sensitive than the HI test (Table 1). On the other hand, the enhanced Nt test gave, on the average, 41-times-higher titers than the HI test and 90-times-higher titers than the conventional Nt test.

TABLE 1. Determination of rubella antibody in immunized children by the HI, conventional Nt, and enhanced Nt tests

Serum no. ^a	Preimmunization titer			Postimmunization titer			Antibody titer ratio		
	HI	Nt	ENT ^b	HI	Nt	ENT ^b	HI/Nt	ENT/NT	ENT/NT
1	<8	<4	<64	128	34	3,000	3.8	23	88
2	<8	<4	<64	128	27	1,400	4.7	11	52
3	<8	<4	<64	64	47	6,500	1.4	102	138
4	<8	<4	<64	64	37	4,500	1.7	70	122
5	<8	<4	<64	128	49	5,000	2.6	39	102
6	<8	<4	<64	128	58	5,800	2.2	45	100
7	<8	<4	<64	32	39	1,200	0.8	38	31
8	<8	<4	<64	128	92	5,200	1.4	41	57
9	<8	<4	<64	64	66	2,700	1.0	42	41
10	<8	<4	<64	128	19	2,300	6.7	18	121
11	<8	<4	<64	128	31	5,600	4.1	44	181
12	<8	<4	<64	256	132	5,800	1.9	23	44
Geometric mean antibody titer or mean antibody ratio				100	46	3,580	2.7	41	90

^a The 12 vaccinees were children 12 months to 6.3 years old (average age, 2.4 years old). These children were immunized with measles-mumps-rubella trivalent vaccine (Merck, Sharp and Dohme). Postimmunization sera were obtained 2 to 5 months after immunization.

^b The enhanced Nt test (ENT) was performed using a 1:3 dilution of AIG.

A comparison of titers in young adults after natural infection and in elderly persons (Table 2) again demonstrated the high degree of potentiation of neutralizing antibody by AIG.

Prozone effect associated with the enhanced Nt test. Table 3 lists the percent virus plaque reduction by sera with different antibody titers. Sera with low titers (no. 3 to 6) showed a

TABLE 2. *Determination of rubella antibody in adult subjects after natural infection*

Serum no. and age group ^a	Antibody titer			Antibody titer ratio		
	HI	Nt	ENT ^b	HI/Nt	ENT/Hi	ENT/Nt
Group A						
1	ND ^c	31	1,300			42
2	ND	130	4,200			32
3	ND	95	5,500			58
4	ND	36	1,300			36
5	ND	100	9,800			98
6	ND	43	410			10
7	ND	71	4,700			66
Geometric mean antibody titer or mean antibody ratio		64	2,568			49
Group B						
8	32	23	1,000	1.4	31	43
9	32	96	3,800	0.3	119	40
10	64	210	5,900	0.3	92	28
11	32	140	3,600	0.2	113	26
12	16	120	5,900	0.1	369	49
13	256	52	2,300	4.9	9	44
14	32	200	10,000	0.2	313	50
15	512	320	17,000	1.6	33	53
16	512	340	8,600	1.5	17	25
17	128	57	5,700	2.3	45	100
Geometric mean antibody titer or mean antibody ratio	78	117	4,966	1.3	114	46

^a Group A were individuals 18 to 32 years old. Group B were individuals 65 years old.

^b The enhanced Nt test (ENT) was performed using a 1:3 dilution of AIG.

^c ND, Not done.

TABLE 3. *Prozone effect associated with AIG-enhanced rubella Nt test*

Test sample	HI titer	Nt titer	Enhanced Nt test										AIG concn	Enhanced Nt titer
			Percent PFU ^a /well at serum dilution (reciprocal):											
			4	16	64	256	1,024	4,096	16,384	65,536	262,000			
Serum														
1	<8	<4	88	100	100	100							Undil. ^b	<64
			100	100	100	100							1:3	<64
2	<8	<4	75	50	83	95							Undil.	22
3	<8	<4	38	58	13	68							Undil.	170
4	8	<4	50	57	4	26	76	100					Undil.	475
5	8	5	35	46	19	100	100	100					1:3	99
			30	56	41	100	93	100					1:5	81
			17	39	47	86	100	100					1:10	82
6	8	8	24	54	11	2	50	91					1:3	1,133
7	128	57	0	11	3	0	3	53	94				1:3	4,112
8	128	153	0	0	0	0	4	31	58	85	100		1:3	11,169
			0	0	0	0	4	59	63	74	81		1:5	10,790
			0	8	19	3	6	67	75	100	100		1:10	4,055
CSF														
1	<8	15	0	0	0	5	36	88	95				1:3	1,480

^a PFU, Plaque-forming units.

^b Undil., Undiluted.

distinct prozone phenomenon, manifested by a lower degree of plaque reduction at dilutions of 1:4 and 1:16 than at dilutions of 1:64. This suggests that the potentiation of the specific antibody-virus complex by AIG was blocked by excessive nonrubella human IgG at low serum dilutions. No prozone effect was observed with CSF, presumably because of its low IgG content.

The resulting antibody titers were clearly dependent on the concentration of the AIG used in the test (Table 3). For economical reasons, titrations utilizing the enhanced Nt test were routinely performed with AIG diluted 1:3 and started at serum dilutions of 1:64. If increased sensitivity was desirable, a 1:16 dilution of serum was included in conjunction with undiluted AIG. The initial CSF dilution was always 1:4 in view of the absence of a prozone phenomenon.

Lack of cross-reactivity of rubella virus and antibody in heterologous tests. Recently we developed sensitive Nt assays for measles (1) and mumps (15) antibodies and have used these together with the rubella Nt test in monitoring the persistence of maternal antibody in infants. It seemed desirable to determine the specificity of each of the three tests in relation to the other two viral antigens.

Table 4 summarizes antibody determinations in three groups of individuals immunized with monovalent rubella, measles, or mumps vaccine. The five young adults who received the rubella

vaccine had preexisting mumps and measles antibody. After rubella immunization their mumps and measles titer did not change significantly. Similarly, immunization of children with monovalent mumps or measles vaccine failed to elicit or boost antibody to the heterologous two antigens. In one instance (serum no. 8), an individual who received measles vaccine did seroconvert simultaneously to mumps virus. With this one exception, the results suggest a lack of shared antigenic determinants detectable by the highly sensitive Nt tests.

Significance of the enhanced rubella Nt test in monitoring antibody in the CNS. Three rhesus monkeys were inoculated intravenously with strain M-33 of natural rubella virus. Antibody titers were monitored in the CSF by the HI, conventional, and enhanced Nt tests. Specific rubella antibody appeared in the CSF of all three animals (Table 5), indicating that antibody leaked across the blood-brain barrier or that rubella infection of the CNS induced local antibody production. No antibody was detected in the CSF by either the HI or the conventional Nt tests.

Detection of early antibody by the enhanced Nt test. Figure 1 shows the antibody response in rhesus monkeys to rubella infection by the HI, conventional Nt, and enhanced Nt tests. The antibody titers followed an expected pattern, peaking at about 3 weeks after infection.

TABLE 4. Specificity of antibody response to rubella, measles, and mumps viruses determined by the sensitive virus plaque Nt tests

Vaccine ^a	Serum no.	Neutralizing antibody titer ^b					
		Rubella		Measles		Mumps	
		Pre	Post	Pre	Post	Pre	Post
Rubella, monovalent	1	<16	1,930	1,250	4,120	1,110	1,310
	2	<16	3,350	240	90	1,200	1,060
	3	<16	5,450	2,120	680	850	580
	4	<16	1,990	260	150	1,770	1,410
	5	<16	2,060	260	380	2,060	1,360
Measles, monovalent	6	<16	<16	<8	1,520	22	22
	7	23	18	<8	750	42	31
	8	<16	<16	<8	980	<16	400
	9	<16	<16	<8	920	21	25
	10	<16	<16	<8	200	26	22
Mumps, monovalent	11	420	460	102	80	<16	2,300
	12	<16	<16	327	230	<16	1,100
	13	<16	<16	1,030	1,120	<16	1,000

^a The rubella vaccine was administered to young adults, the measles vaccine was administered to infants 12 months of age, and the mumps vaccine was administered to children of 4 to 8 years. Convalescent sera were obtained 4 to 6 weeks after immunization.

^b Titer was determined before (Pre) and after (Post) immunization. Rubella and mumps antibodies were determined by the enhanced Nt test. Serum titration was started at a dilution of 1:16. AIG was used undiluted for serum dilutions of 1:16 and at 1:3 for serum dilutions $\geq 1:64$. Measles antibody was determined by the conventional Nt test.

TABLE 5. *Specific antibody in the serum and CSF of rhesus monkeys infected intravenously with natural rubella virus*

Monkey no.	Specimen	Antibody test	Enhanced Nt titer at weeks after infection:						
			0	1	2	3	4	6	8
350	CSF	HI	<4	<4	ND ^a	ND	ND	<4	<4
		Nt	<4	<4	ND	ND	ND	<4	<4
		ENt ^b	<4	<4	ND	ND	ND	33	53
	Serum	ENt	<64	<64	7,400	60,000	23,000	9,000	10,000
		CSF/serum anti-body ratio ^c						3.7	5.3
420	CSF	HI	<4	<4	<4	ND	<4	<4	<4
		Nt	<4	<4	<4	ND	<4	<4	<4
		ENt	<4	<4	<4	ND	9	9	8
	Serum	ENt	<64	<64	13,000	44,000	19,000	38,000	12,000
		CSF/serum anti-body ratio					0.5	0.2	0.7
558	CSF	HI	<4	<4	<4	<4	<4	<4	ND
		Nt	<4	<4	<4	<4	<4	<4	ND
		ENt	<4	<4	7	42	34	341	ND
	Serum	ENt	<64	<64	9,200	24,000	32,000	34,000	8,000
		CSF/serum anti-body ratio			0.8	1.8	1.1	10.0	

^a ND, Not done.^b ENt, enhanced Nt test. A 1:3 dilution of AIG was used in this study.^c (Enhanced Nt titer in the CSF/enhanced Nt titer in the serum) × 1,000; the normal range of CSF/serum antibody ratio determined in 10 rhesus monkeys was 0.2 to 0.8. FIG. 1. *Antibody response in rhesus monkeys infected with natural rubella virus intravenously. Virus isolation was attempted from throat swab. —, Negative; +, positive virus isolation.*

AIG-potentiased Nt antibody titers were, on the average, 24 to 93 times higher than HI antibody and 151 to 331 times higher than nonenhanced

Nt antibody. Ten days after infection, while virus was still present in the throats of the animals, antibodies were detected in the serum by the enhanced Nt test but not by the other two antibody assays.

DISCUSSION

Previous tests for measuring rubella Nt antibody used several techniques to indicate virus neutralization, e.g., viral interference (11), inhibition of direct cytopathic effect (8), or reduction of plaque formation (14). However, all three of these assays are less sensitive than the rubella HI test (16). As a consequence, the HI test, convenient and relatively sensitive, remains the routine test for measuring rubella antibody.

There are instances in which a considerably higher degree of sensitivity is required than is provided by the HI or Nt tests. These instances involve the detection of low antibody titers in the CSF in instances of subclinical or suspected latent CNS infections or the question of the persistence of maternal antibody in infants and its effect on immunization with attenuated vaccines. The first situation is exemplified by the CSF titration result listed in Table 3. The CSF was from a child with congenital rubella in whom the high CSF antibody titer and a CSF/serum antibody ratio of 14.7 (normal range 1.0 to 6.0) indicated CNS involvement. The HI test was negative and failed to suggest CNS involvement.

Similarly, the three rhesus monkeys infected with natural rubella virus (Table 5) developed CSF antibody titers demonstrable only by the enhanced Nt test. Animals no. 350 and 558 developed a CSF titer and a CSF/serum antibody ratio of more than 0.8 (normal range is 0.2 to 0.8; see Table 5 and ref. 5). The CSF/serum antibody ratio for influenza virus measured in the same CSF and serum samples was less than 0.8 (not shown). These results indicate that in monkeys no. 350 and 558 rubella virus invaded the CNS tissue and stimulated local antibody production. Thus, measuring CSF viral antibody by sensitive techniques represents a convenient way to determine neuroinvasiveness and neurotropism of viral agents in experimental or natural infections.

Another area in which the enhanced rubella Nt test was applied concerns the persistence of maternal antibody in infants during the first year of life. A study performed in collaboration with David W. Reynolds (Department of Pediatrics, University of Alabama, Birmingham, Ala.), to be reported soon, has shown that rubella antibody remains in a certain proportion of infants in measurable titers up to 12 months of age. In most instances, however, this persisting antibody does not interfere with successful immunization.

A comparison of absolute rubella titers in a group of randomly selected elderly people, young adults, and immunized children (Tables 1 and 2) revealed that the titers were higher in the elderly group of people, probably as a result of boosting in repeated rubella epidemics. On the other hand, potentiation of Nt antibody by AIG was more effective in children shortly after immunization (90-fold average increase) than in the two adult groups (49-fold and 46-fold average increase, respectively). It has been suggested that AIG enhances virus Nt by firming up the specific virus-antibody complex and/or by adding an additional layer of protein around the sensitized virus (2). Early antibody is known to have lower affinity for the homologous antigen than late antibody (19), and this difference may account for the higher degree of potentiation of the early postimmunization sera in children as opposed to the sera from adults. In accordance with this interpretation is the even higher degree of potentiation in the very early sera of monkeys infected with non-attenuated rubella virus.

The pronounced effect of AIG on reducing the infectivity of sensitized rubella virus prompted us to examine whether a similar potentiation could be observed in the rubella HI test. The procedure described in Materials and Methods was modified in that the rubella hemagglutinin-

antibody mixture was reacted with 1:3-diluted AIG before erythrocytes were added. Virtually no enhancement of HI antibody was observed, suggesting that the mechanisms of virus neutralization and antigen-induced hemagglutination are basically different.

In a previous study dealing with mumps Nt antibody, it was shown that specific IgG or IgM antibody could be potentiated by AIG to approximately the same extent (15). Although not tested in the present study, it can be assumed that the enhanced rubella Nt test will provide an equally sensitive and specific distinction of the two classes of antibody, an important criterion in distinguishing primary from secondary rubella infection.

Other techniques, with increased sensitivity in measuring rubella antibody, have been described. The radioimmunoassay was approximately 15 times more sensitive than the HI test (9), and an enzyme immunoassay (4) compared favorably with the HI test. A question of considerable importance in utilizing tests of increased sensitivity concerns the degree to which they may cross-react with antigenically related agents. In general, measuring of antibody directed to the viral envelope, as is the case in the enhanced Nt test, may be less cross-reactive than assays which measure antibody to both internal and external viral components. The high degree of specificity of the very sensitive enhanced Nt test for rubella, mumps, and measles antibody was established by testing the immune response to immunization with monovalent rubella, mumps, or measles vaccines. The recipients responded with high Nt antibody titers to the homologous antigen, whereas their antibody titers to the other two viral antigens, with one exception, remained unchanged. This finding is important in monitoring the response to the trivalent measles-mumps-rubella vaccine which is being used with increased frequency in immunization programs in early childhood.

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