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Rubella Reimmunization: Comparative Analysis of the Immunoglobulin G Response to Rubella Virus Vaccine in Previously Seronegative and Seropositive Individuals

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Rubella virus (RV)-specific immunoglobulin G (IgG) antibodies were studied in military recruits undergoing unselected immunization with live attenuated measles, mumps, and rubella virus (MMR) vaccine. Three different whole-RV enzyme immunoassays (EIAs) and an epitope-specific EIA with a synthetic peptide (BCH-178c) representing a neutralization domain on the RV E1 envelope protein were used. Before vaccination, 84.2, 87.7, and 84.5% of the subjects tested ($n = 399$) were found to be seropositive (>10 IU/ml or assay equivalent) by the three whole-RV EIAs, respectively, while only 82.5% were seropositive by the BCH-178c EIA. Although prevaccination seropositivity rates were similar for the whole-RV EIAs (sensitivity, 94 to 100%), many sera considered seropositive by the whole-RV EIAs had E1 peptide EIA antibody levels of <10 IU/ml (sensitivity, 77.4 to 80.7%). One month after vaccination, 97.8, 97.2, and 93.5% of the subjects who were followed ($n = 356$) were seropositive by the three whole-RV EIAs, respectively, while 89% had BCH-178c peptide-specific IgG titers of >10 IU/ml. After vaccination, depending on the assay used, up to 20.6% of initially seropositive individuals exhibited a greater than fourfold increase in RV-specific IgG, while up to 47.3% showed a greater than twofold increase. Increased antibody titers after vaccination (seroboosting) were most frequently associated with low levels of BCH-178c peptide-specific IgG before vaccination. RV protein-specific IgG was also studied by immunoblot assays in a subset ($n = 56$) of individuals receiving the MMR vaccine. Of these, 89.4 and 91.1% exhibited RV protein (E1, E2, and C protein)-specific IgG before and after vaccination, respectively. Seroboosting (two- to fourfold increase in EIA titers of individuals seropositive by the whole-RV EIA before vaccination) was usually accompanied by a shift in the IgG immunoblot pattern from a single (E1) to multiple (E1-E1, E1-C, or E1-E2-C) specificities, suggesting exposure of new epitopes as a result of viral replication.

Natural rubella virus (RV) infection is characterized by fever, sore throat, lymphadenopathy, and skin rash which usually resolve quickly (42). Because these symptoms are also sequelae of several other common viral infections, clinical rubella is difficult to diagnose (3, 32). It has been estimated that 30 to 50% of RV infections are clinically inapparent (1, 2) and would only be detected serologically (5, 9, 10). Since maternal infection during the first trimester may result in spontaneous abortion or in fetal infection, leading to serious birth defects (congenital rubella syndrome [CRS]) (25), it has become routine practice to offer immunization to all RV-seronegative women of childbearing age as well as to infants ages 12 to 15 months. When such vaccination programs have been undertaken, the incidence of reported rubella and CRS has declined markedly (5). However, rubella has not been completely eradicated and outbreaks occur at regular intervals. These have been associated with either clinical or subclinical infections in pregnant women and fetal infections (5–7, 10, 21). Although initial seroconversion rates of >95% were reported in RV vaccine efficacy studies (21, 22, 35), serologic surveys have indicated that 6 to 25% of women of childbearing age may be RV seronegative and therefore at risk of infection (7, 14, 26, 36). There is now ample evidence that RV reinfection occurs more frequently in vaccinated than in naturally immune individuals (6), and the increasing number of reports of rubella in adolescents who were vaccinated in infancy (39) also attests to RV vaccine failure. Anecdotal reports of rubella reinfection occurring in RV-seropositive individuals (5–7, 10) and observations of virus shedding and increased levels of RV-specific immunoglobulin G (IgG) (seroboosting) (9, 16, 20, 31, 34, 36) suggest that such individuals may lack critical components of rubella immunity that would otherwise protect them from virus challenge and viremia.

The normal primary immune response to RV is characterized by a transient rise in RV-specific IgM antibodies, which is followed by a rise in IgG antibodies, which are usually detectable from the fourth week onward. Secondary immune responses are generally characterized by an increase in RV-specific IgG and, occasionally, the transient appearance of specific IgM (3, 32). The observation of a fourfold or greater increase in the titers of RV-specific IgG in paired acute- and convalescent-phase serum specimens is considered confirmatory for RV infection (32). Although virus neutralizing (NT)

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antibodies are associated with protective immunity, these are rarely evaluated. Instead, RV-specific antibodies have been measured by single radial hemolysis, complement fixation, hemagglutination inhibition (HAI), and latex agglutination assays and more recently by enzyme immunoassays (EIAs) with whole RV. Currently, titers of RV-specific IgG of ≥1:8 detected by HAI assay or levels of ≥10 IU/ml determined by EIA are considered protective (32).

RV has three major antigenic proteins: the envelope glycoproteins E1 and E2 and the internal capsid protein C. The protein most characterized biologically is E1, which contains four NT domains as well as the target sequence for HAI antibodies (8, 40, 43) located between amino acid residues 209 and 291. Synthetic peptides have been used to map one NT domain located between E1 residues 213 and 239 recognized by both murine and human antibodies (29). Those investigations have indicated that after RV infection or vaccination, antibodies directed to E1 (residues 213 to 239) increase in parallel and correlate with the RV-specific antibodies measured by other EIAs and by HAI and NT tests (29, 46). The E1 NT domain peptide-specific antibodies are lacking in individuals who remain seronegative, despite multiple RV vaccinations, who have been exposed to RV by congenital infection (29), or who show adverse reactivity to RV such as arthritis associated with persistent RV infection (27, 28). The present study concerning unselected rubella vaccination of Canadian military personnel provided the means to evaluate the capacities of the whole-RV EIAs currently used in rubella antibody screening programs and the E1 NT domain peptide EIA (the E1 EIA) to predict protection against virus challenge.

MATERIALS AND METHODS

Study subjects and serum samples. Study subjects were Canadian military recruits (n = 399) based in three separate geographic areas and included 356 males (ages 17 to 44 years; median age, 20 years), 36 females (ages 18 to 33 years; median age, 20 years), and 7 subjects for whom gender and age were not recorded. Histories of previous rubella disease or vaccination were not obtained. All recruits were vaccinated parenterally with a single dose of live attenuated measles, mumps, and rubella virus (MMR) vaccine (MMR II; Merck Ltd.). According to the manufacturer's specifications this vaccine contains no less than 10^3 PFU of live attenuated RA27/3 strain RV per dose. Serum samples were collected just before and at 1 month after vaccination. Samples were divided into aliquots, frozen, and then distributed to three different centers for various aspects of rubella and measles antibody testing: the Laboratory Centre for Disease Control in Ottawa, Ontario, Canada (study coordination); the Alberta Provincial Virology Laboratory in Edmonton, Alberta, Canada (measles virus antibody determinations and RV antibody screening by Behring Enzygnost Rubella IgG EIA); and the University of British Columbia in Vancouver, British Columbia, Canada (for the detailed analysis of RV-specific antibodies, which is the subject of this report). The results of measles antibody testing of these sera have been reported elsewhere (12).

Whole-RV EIAs. Total quantitative RV-specific IgG levels were measured by three different EIAs. These included Diamedix Rubella IgG Microassay (Diamedix EIA; Diamedix Corporation, Miami, Fla.) and the Behring Enzygnost Rubella IgG EIA (Behring EIA; Behringwerke, Marburg, Germany), both of which are widely used in rubella antibody screening programs. At the time of the present study, the Diamedix EIA used the purified Gilchrist strain of RV as the target antigen for detecting RV-specific IgG, while the Behring EIA uses a cell lysate from BHK-21 cells infected with an unspecified strain of RV. Sera were also tested by an in-house rubella IgG EIA (41) which uses purified detergent-solubilized RV (M33 strain) grown on Vero cells (referred to as the M33 EIA). All EIAs were standardized against the World Health Organization (WHO) Rubella IgG reference standard serum sample (44).

(i) Diamedix EIA. Sera were tested at a dilution of 1:40 according to the manufacturer's instructions. The results were expressed in EIA units (EU) per milliliter by comparison of the absorbance value obtained with the test serum with that obtained with the manufacturer's reference serum. The cutoff for this assay has been designated 15 EU/ml, which is equivalent to 10 IU/ml on the basis of comparisons with the WHO rubella IgG reference standard serum and the Centers for Disease Control and Prevention's rubella IgG reference serum (24a).

(ii) Behring EIA. Sera were tested at a dilution of 1:204. The results were expressed as net absorbance (A_{405}) units after subtraction of the background absorbance measured for negative control wells (coated with lysate from noninfected BHK-21 cells). The cutoff for this assay was 0.200 net absorbance units, which was considered equivalent to an HAI titer of 1:8 or 10 IU/ml relative to the WHO rubella IgG reference standard.

(iii) M33 EIA. The M33 EIA is fully quantitative and uses a five-point standard curve (0.0625 to 1 IU/ml) with a pooled serum reference which has been assigned units relative to the WHO rubella IgG reference standard. Sera were tested at four dilutions (1:100 to 1:800). The results (in international units per milliliter) were computed from the measured absorbance values of at least three test serum dilutions by extrapolation from linear regression standard curves prepared for each assay plate. Negative control sera routinely showed absorbance values of less than 0.100 units. Interassay variance was <15%. The negative cutoff for this assay was taken to be 10 IU/ml to allow direct comparison with the results obtained by the other EIAs.

Synthetic peptide EIA. Specific IgG directed to an RV E1 protein NT domain were measured by a commercial EIA (DETECT-RUBELLA G; BioChem Immunosystems, Inc., Montreal, Quebec, Canada), which uses a cyclized synthetic peptide, BHC-178c (29, 46) representing E1 residues 213 to 239. This region is conserved among all known strains of RV, including the HPV77/DE5 and RA27/3 vaccine strains. Sera were diluted to 1:51 and were tested according to the manufacturer's directions. The results were expressed in international units per milliliter after conversion of the ratio of the absorbance measured for the serum sample to the absorbance measured for the reference standard included with the kit. Although the biological limits of immune protection have not yet been established for the E1 peptide EIA, this assay has also been standardized against the WHO rubella IgG reference standard. Hence, the cutoff was taken to be 10 IU/ml for direct comparison with the results obtained by the other EIA methods.

Immunoblot assays. RV protein-specific IgG and IgM antibodies were determined by nonreducing immunoblot assays (45). IgG antibodies were tested at a 1:50 dilution, while IgM antibodies were tested at a dilution of 1:10 after removal of competing RV-specific IgG and rheumatoid factor by preadsorption of the sera with protein G (45). For each study subject evaluated for RV protein-specific IgG, pre- and postvaccination sera were run in parallel on the same immunoblot, while IgM immunoblots were performed with postvaccination sera only. A positive result was recorded as the appearance of a band at the known molecular positions of the RV E1, E2, and C proteins in comparison with the positions of the bands of a positive control serum pool run on each immunoblot. The negative control sera run on each immunoblot showed no bands.

Study assumptions and analysis of results. Because three of the EIA methods used in the present study were single-point assays and used different antibody dilutions, direct comparisons of quantitative levels of rubella antibody in international units or EU per milliliter were not made. However, since the cutoff for each method was established by using the same international (WHO) reference standard, it was possible to compare the relative numbers of seropositive or seronegative subjects on the basis of the assumption that the EIA methods were equivalent at their established cutoff points. The results were also expressed as a postvaccination/prevaccination ratio by dividing the antibody level measured in the postvaccination sample by that measured in the prevaccination serum sample. Within- and between-assay comparisons were also made by using postvaccination/prevaccination ratios to determine the frequency of serologic boosting (two- or fourfold or greater increases in postvaccination titers). Postvaccination/prevaccination ratios were also used to examine the relationships between the pre- and postvaccination antibody levels measured by the various methods. Because the MMR vaccine contains approximately 10^3 PFU of the live attenuated RA27/3 strain RV per dose, the assumption in the present study was that significant increases (two- to fourfold or greater) in postvaccination titers would be observed only if viral replication had occurred (19). Thus, a postvaccination/prevaccination ratio of ≥4 was taken as an indicator of RV vaccine-induced viremia (3, 33) by applying the same criteria used to define clinical infection with wild-type RV (32). Pre- and postvaccination and age group differences in antibody levels were compared by both parametric (Student's t test) and nonparametric (Kruskall-Wallis, Wilcoxon signed-rank, and Mann Whitney tests) methods.

RESULTS

Serologic status pre- and postvaccination with MMR vaccine determined by EIAs. Table 1 summarizes the pre- and postvaccination serologic status of the vaccinees determined by whole-virus and E1 (BCH-178c peptide) EIAs. Before vaccination, most of the study participants were well within the seropositivity ranges by all methods. Because the MMR vaccine was introduced in 1969 and 1970 for routine use for infant immunizations, the levels of RV-specific IgG in subjects 21 years or younger (n = 209) and greater than 21 years (n = 169) of age were compared to determine if the evaluation methods used in the present study would detect differences between subjects who were likely to have been vaccinated as infants and older individuals who had likely acquired their rubella immunity through natural infection. With the exception of the E1

TABLE 1. RV serologic status determined by EIAs pre- and postvaccination with MMR vaccine

EIA	Prevaccination (n = 399)		Postvaccination (n = 356)		Ratio (%)[c]	
	No. (%) seropositive[a]	Antibody level (GMT [median])[b]	No. (%) seropositive[a]	Antibody level (GMT [median])[b]	≥2	≥4
Diamedix	336 (84.2)	35.2 (45.7)	348 (97.8)	52.1 (58.3)	81 (22.3)	31 (8.7)
Behring	350 (87.7)	0.716 (1.150)	346 (97.2)	1.403 (1.584)	98 (27.5)	48 (13.5)
M33	337 (84.5)	60 (96.6)	333 (93.5)	152.4 (214.2)	163 (45.8)	82 (23)
E1	329 (82.5)	20.8 (20.7)	317 (89.0)	45.9 (53)	126 (35.4)	83 (23.3)

[a] Diamedix EIA negative cutoff, <15 EU/mL; Behring EIA negative cutoff, <0.200 A_{405} units; M33 EIA negative cutoff, <10 IU/ml; and E1 peptide EIA negative cutoff, <10 IU/ml.
[b] GMTs are in EU per milliliter (Diamedix EIA), A_{405} units (Behring EIA), or international units per milliliter (M33 and E1 EIAs).
[c] Postvaccination/prevaccination ratio (see Materials and Methods).

(BCH-178c peptide) EIA, mean and median prevaccination antibody levels (data not shown) were found to be significantly higher in the older age group (Diamedix EIA, $P < 0.0001$; Behring EIA, $P = 0.014$; M33 EIA, $P = 0.013$; E1 EIA, $P = 0.062$). No significant age-related differences were observed in postvaccination antibody levels. One month after receiving the MMR vaccine, only 8 of 356 (2.3%) subjects were found to be seronegative by the Diamedix and Behring EIAs. These included four individuals who were initially seronegative and who remained seronegative after vaccination, as determined by all evaluation methods (vaccine failures), and four who after vaccination became borderline seropositive, as determined by at least one method. The percentages of subjects considered seronegative by the M33 and E1 EIAs after vaccination were higher, being 5.9 and 9.9%, respectively. Although Table 1 also provides the geometric mean titer (GMT) and the median pre- and postvaccination levels of RV-specific IgG, because of the methodologic limitations of the single-point EIAs, total quantitative levels were not statistically compared between assays. Also shown in Table 1 are the percentages of subjects exhibiting a serologic boost in RV-specific IgG following vaccination with the MMR vaccine. The highest postvaccination/prevaccination ratios were often observed in subjects who were either initially seronegative or who otherwise had relatively low prevaccination levels of RV-specific IgG (see Fig. 1 and 2).

The sensitivities and specificities of the four EIA methods were compared by using the cutoff ranges indicated above (Table 2). Although the pre- and postvaccination sensitivities of the whole-RV EIAs were comparable, this was not the case with the E1 EIA results, by which a substantial proportion of the subjects shown to be seropositive by the former methods actually had low levels (<10 IU/ml) of E1 peptide-specific IgG. This was also reflected in the low specificities that were observed (Table 2).

Serologic response to vaccine relative to prevaccination antibody levels determined by EIAs. To estimate the ability of each EIA method to predict immune protection, i.e., protection versus vaccine-induced viremia (see the section above describing study assumptions), the postvaccination/prevaccination ratios calculated for each method were compared with the prevaccination antibody levels determined by each EIA. This approach was designed to reveal how useful each assay cutoff point might be in determining whether or not a given individual would seroboost (become viremic) in response to the vaccine and also how sensitive each method was in detecting changes in postvaccination antibody levels. The relative proportions of the study subjects with postvaccination/prevaccination ratios of ≥2 and ≥4 were compared.

The Behring EIA is widely used in rubella antibody screening programs designed to advise women of childbearing age about their immune status. By using a cutoff point of 0.200 absorbance units (equivalent to 10 IU/ml), the postvaccination/prevaccination ratios determined by the four EIA methods were compared for subjects who were considered to be seronegative or seropositive before vaccination (Fig. 1). Within-assay comparisons (Fig. 1) revealed that not only seronegative subjects but also seropositive subjects with prevaccination titers as high as 1.000 unit by the Behring EIA could seroboost (fourfold or greater increase). Similarly, subjects who would have been considered seropositive by the Behring EIA before vaccination were also observed to have substantial increases in postvaccination antibody levels measured by the Diamedix, M33, and E1 EIAs.

Prevaccination serologic status measured by the E1 peptide EIA was compared with the postvaccination/prevaccination ratios measured by each of the other EIA methods (Fig. 2). An arbitrary cutoff of 10 IU/ml was used to compare subjects who would have been considered seropositive or seronegative on the basis of their prevaccination E1 peptide EIA results. As can be seen from the data presented in Fig. 2, the E1 EIA appeared to be somewhat more predictive of the serologic response to vaccine strain RV in that relatively few subjects with prevaccination titers of >10 to 20 IU/ml exhibited a booster response as measured by the Behring, Diamedix, and

TABLE 2. Sensitivities and specificities of EIAs for determination of RV-specific IgG

EIA	Sensitivity (specificity)[a]							
	Diamedix EIA		Behring EIA		M33 EIA		E1 peptide EIA	
	Prevaccination	Postvaccination	Prevaccination	Postvaccination	Prevaccination	Postvaccination	Prevaccination	Postvaccination
Diamedix	100 (100)	100 (100)	94 (85.7)	99.4 (75)	97.3 (89.1)	96.1 (38.1)	97.9 (58)	90.5 (14.3)
Behring	97.9 (66.7)	99.4 (75)	100 (100)	100 (100)	99.4 (70.9)	96.1 (38.1)	99.4 (44.9)	90.2 (11.4)
M33	98.2 (84.5)	96.3 (100)	95.4 (90.7)	96.2 (100)	100 (100)	100 (100)	98.8 (56.5)	90.5 (14.3)
E1	80.7 (90.5)	91.4 (62.5)	77.4 (91.8)	91 (50)	80.1 (92.7)	95.2 (23.8)	100 (100)	100 (100)

[a] Units for both sensitivity and specificity are percent. Sensitivity, percentage of subjects in the group who were considered seropositive by both methods indicated in each field; specificity, percentage of subjects who were considered seronegative by both methods indicated in each field.

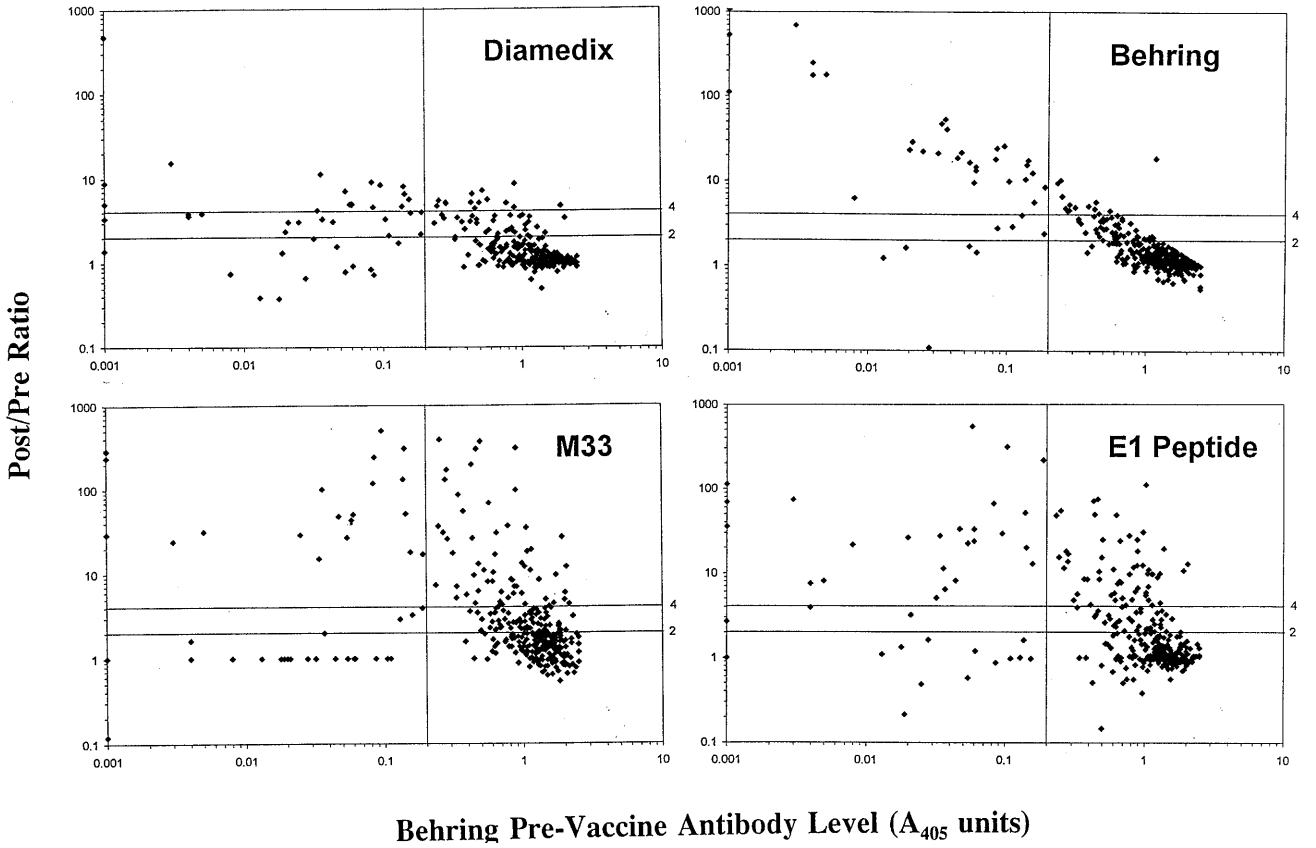

FIG. 1. Serologic response relative to prevaccination antibody levels determined by Behring EIA. The serologic responses (postvaccination/prevaccination ratio; *y* axis) determined by the Diamedix, Behring, M33, and E1 EIAs, relative to prevaccination antibody levels (in net absorbance units) determined by the Behring EIA (*x* axis), are shown. The negative cutoff for the Behring EIA (0.200 units) and postvaccination/prevaccination ratio intervals of 2 and 4 are indicated.

E1 EIAs. However, the prevaccination titer by the E1 peptide EIA was less predictive of the serologic responses measured by the M33 EIA.

The postvaccination/prevaccination ratios determined by each of the EIA methods were compared for subjects whose prevaccination antibody determinations indicated that they were initially seropositive. For example, the postvaccination/prevaccination ratios determined by the Diamedix, Behring, M33, and E1 EIAs were compared for the 300 subjects whose prevaccination titers as measured by the Diamedix EIA exceeded 15 EU/ml (Table 3). Similarly, postvaccination/prevaccination ratios were compared for subjects found to be initially seropositive by the Behring, M33, and E1 EIAs (Table 3). Although the number of initially seropositive subjects exhibiting large (fourfold or greater increases in RV antibody levels after vaccination with the MMR vaccine determined by the Diamedix and Behring EIAs were small, the numbers of initially seropositive individuals who exhibited fourfold or greater increases detected by the M33 and E1 EIAs were considerably larger. Hence, the negative cutoff limits used for each EIA method appeared to be too low for predicting immune protection (no seroboosting) as evaluated by the surrogate approach.

RV protein-specific IgM and IgG antibodies determined by immunoblot assays. The synthesis of RV-specific IgM (followed later by the synthesis of specific IgG) in previously seronegative subjects has long been used as a diagnostic indicator of a primary response to RV, while a rapid fourfold or greater increase in RV-specific IgG in a previously exposed or immunized individual may be considered indicative of RV reinfection (3, 32). Therefore, in an attempt to distinguish primary from secondary immune responses in challenge infections with the live attenuated vaccine strain of RV, RV protein-specific IgM antibodies in a randomly chosen subset of postvaccination samples from 193 subjects were evaluated by immunoblot assays. To improve the sensitivity of detection and to eliminate interference from competitive high-avidity RV-specific IgG or from rheumatoid factor, these assays were performed under nonreducing conditions after first removing all IgG antibodies by pretreating the sera with protein G-Sepharose (45). Of the 193 post-MMR vaccination serum samples tested, 62 (32.1%) were found to contain RV-specific IgM. Of these, many also had RV-specific IgG antibody, as determined by the EIAs and IgG immunoblot assays with serum samples obtained both before and after immunization with the MMR vaccine (data not shown).

Pre- and postvaccination sera from a smaller group of individuals ($n = 56$) were tested by all methods, including the four EIAs and both IgM and IgG immunoblot assays. Within this subgroup, 5 of 56 of these subjects were determined to be vaccine failures; i.e., both before and after vaccination their samples were seronegative by all methods. One month after vaccination, 25 of 56 (44.6%) subjects had RV protein-specific IgM in their sera. When the IgG responses were compared, it was observed that 50 of 56 (89.3%) and 51 of 56 (91.1%) subjects had RV protein-specific IgG, as demonstrated by nonreducing immunoblots performed with their pre- and postvaccination sera, respectively. Of the 51 subjects who were IgG immunoblot positive after vaccination, 15 had exhibited a shift

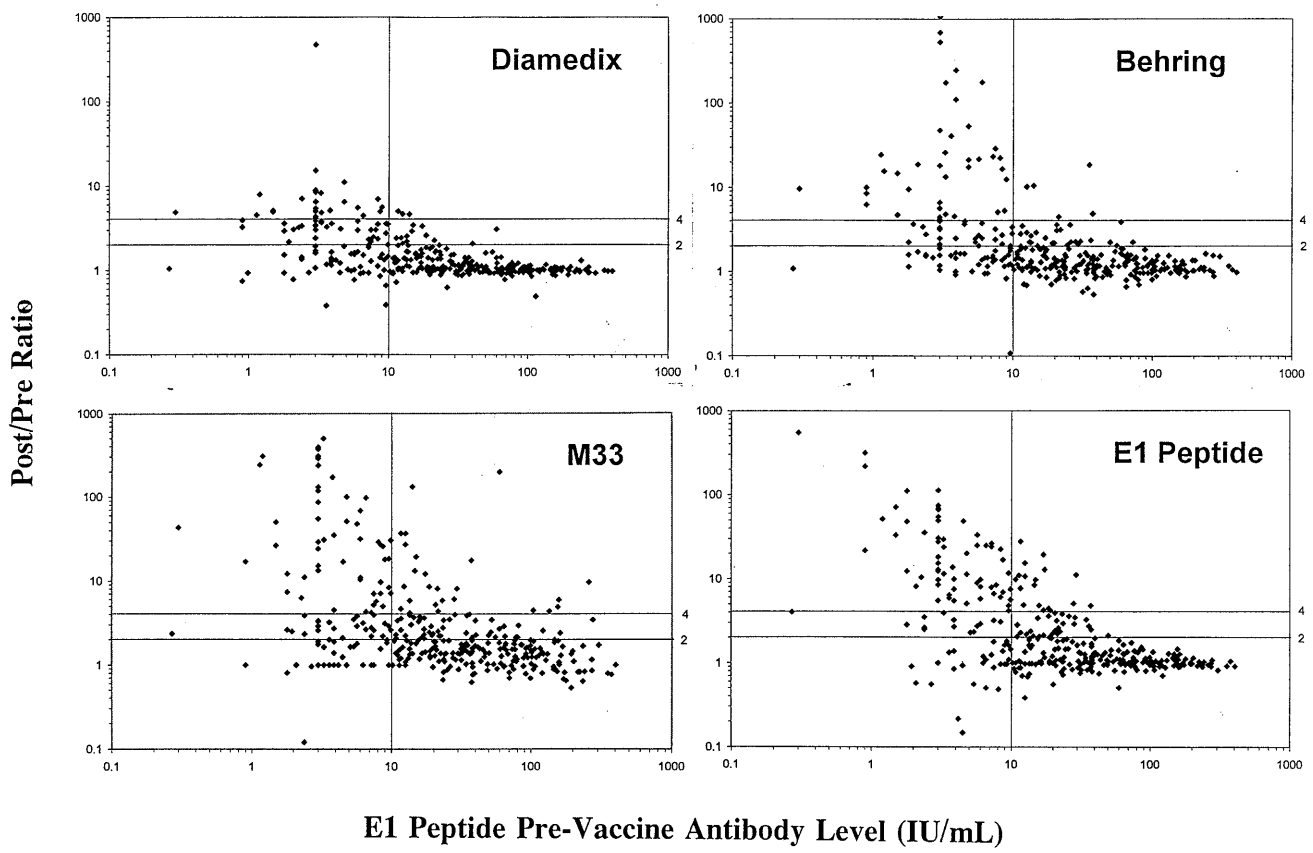

FIG. 2. Serologic response relative to prevaccination antibody levels determined by E1 EIA. Serologic responses (postvaccination/prevaccination ratios; *y* axis) determined by the Diamedix, Behring, M33, and E1 EIAs, each relative to prevaccination antibody levels (in international units per milliliter) determined by the E1 EIA (*x* axis), are shown. The arbitrary negative cutoff for the E1 EIA (10 IU/ml) and postvaccination/prevaccination ratio intervals of 2 and 4 are indicated.

to a more complex pattern of antibody specificities, while the remainder showed no change in protein banding patterns (Fig. 3). Moreover, sera from 14 of 15 of these subjects had E1 peptide antibody levels of <10 IU/ml before vaccination, while only 2 of these serum samples were considered seronegative by whole-virus EIAs. After vaccination, 9 of 15 subjects had both RV protein-specific IgM and IgG antibodies. Table 4 summarizes the pre- and post-MMR vaccination RV antibody levels (GMT) and serologic outcomes (postvaccination/prevaccination ratios) by method in relation to whether or not a shift in the immunoblot pattern was observed after immunization with the MMR vaccine. Overall, prevaccination titers were significantly lower and postvaccination/prevaccination ratios were higher for subjects who exhibited a shift in immunoblot patterns. Thus, in a proportion of the study subjects, RV protein-specific IgM and IgG antibodies were coordinately expressed; 53% of the subjects demonstrating IgM synthesis with a shift in the IgG immunoblot pattern were initially seronegative by the E1 peptide EIA, whereas 13.5% of the subjects did not exhibit this type of vaccine response (data not shown). Moreover, 7.1, 13.3, 46.7, and 53.3% of these subjects had postvaccination/prevaccination ratios of ≥4 by the Diamedix, Behring, M33, and E1 peptide EIAs, respectively, whereas 0, 2.7, 21.6, and 5.4% of the subjects did not show the same response pattern by the same methods, respectively. Finally, all but four of the subjects exhibiting a postvaccination shift in the IgG immunoblot profile lacked RV E2-specific IgG in their prevaccination samples, in contrast to those individuals who did not show these changes (Fig. 3). Thus, a lack of E2-specific IgG before vaccination was associated with both a shift to a more complex immunoblot pattern and seroboosting (as detected by EIA) except in four individuals who displayed an E1 and C pattern both before and after vaccination with the MMR vaccine. Those four individuals had high prevaccination levels of E1 peptide-specific IgG.

TABLE 3. Serologic outcome of rubella immunization for individuals considered seropositive before vaccination determined by whole-RV and RV peptide EIAs

EIA used for prevaccination antibody determination	No. of seropositive subjects identified and compared[a]	No. (%) of subjects with postvaccination/prevaccination ratios of ≥4 by assay method			
		Diamedix EIA	Behring EIA	M33 EIA	E1 peptide EIA
Diamedix	300	2 (0.7)	10 (3.3)	51 (17)	47 (15.7)
Behring	312	14 (4.5)	15 (4.8)	64 (20.5)	59 (18.9)
M33	301	9 (3)	11 (3.7)	44 (14.6)	50 (16.6)
E1	247	4 (1.6)	5 (2)	30 (12.2)	19 (7.7)

[a] Subjects with prevaccination antibody levels greater than or equal to 15 EU/ml (Diamedix EIA), 0.200 net absorbance units (Behring EIA), 10 IU/ml (M33 EIA), or 10 IU/ml (E1 EIA) by the indicated EIA method used for prevaccination serologic evaluation.

DISCUSSION

The primary objective of the present study was to evaluate the serologic status of young Canadian adults to rubella and

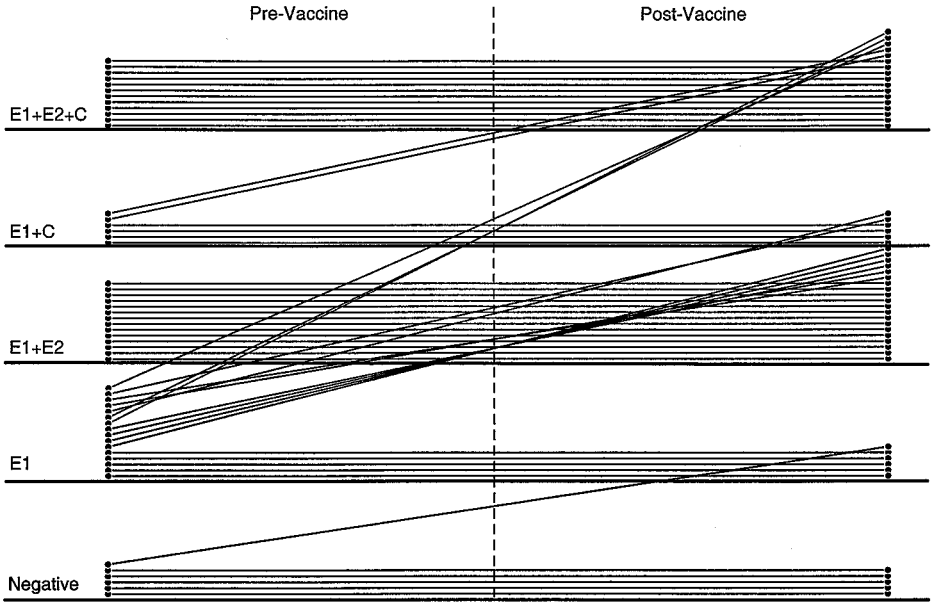

FIG. 3. Comparative immunoblot patterns before and after vaccination against RV. IgG immunoblot assays were performed under nonreducing conditions with the M33 strain of RV as the antigen. RV protein (protein E1, E2, and C)-specific bands were identified according to their known positions relative to molecular weight markers and in comparison with bands observed with a positive control serum sample containing all specificities for all three proteins. Only qualitative results (protein specificities observed in pre- and postvaccination sera from 56 subjects given the MMR vaccine) are shown.

measles viruses. Many of these individuals were likely to have been immunized with the MMR vaccine in childhood. Measurement of RV-specific IgG in prevaccination serum samples from 399 military recruits aged 17 to 44 years (median age, 20 years) about to undergo nonselective vaccination with the MMR vaccine revealed seropositivity rates of 84.2, 87.7, 86, and 82.7% by the Diamedix, Behring, M33, and E1 (BCH-178c) EIAs, respectively. Determination of RV protein-specific IgG by nonreducing immunoblot assays in the prevaccination sera from 56 of these subjects indicated that 89.4% were seropositive by this method. Although rubella vaccine and disease histories were not available, the prevaccination antibody levels measured by these EIAs were shown to be significantly higher in study subjects >21 years of age, who were likely to have become immune as a result of natural rubella infection rather than by vaccination.

One month after receiving a single dose of the MMR vaccine, which contained approximately 10^3 PFU of the live attenuated RA27/3 strain of RV, 97.8, 97.7, 94.1, and 90.1% of the 356 subjects followed were seropositive by the Diamedix, Behring, M33, and E1 EIAs, respectively, while 91.1% of the 56 subjects studied by immunoblotting had RV protein-specific IgG. The Diamedix and Behring EIAs (both of which have been clinically validated and are widely used in rubella antibody screening programs) and the M33 EIA use whole RV. The E1 (BCH-178c) peptide EIA, which is not yet in routine clinical use, measures antibody directed to an NT domain on the RV E1 envelope protein encompassing amino acids 213 to

TABLE 4. Immunoblot response patterns in relation to pre- and postvaccination RV antibody levels determined by EIA

EIA method compared	Immunoblot pattern after vaccination	No. of subjects	Antibody level (GMT)		Postvaccination/ prevaccination ratio
			Prevaccination	Postvaccination	
Diamedix	Change	15	33.6 ($P = 0.0022$)[a]	58.4 ($P = 0.4303$)	1.7 ($P = 0.0005$)
	No change	36	53.5	61.2	1.1
	Negative	5	12.6	9.9	0.8
Behring	Change	15	0.800 ($P = 0.0080$)	1.444 ($P = 0.3969$)	1.8 ($P = 0.0036$)
	No change	36	1.276	1.513	1.2
	Negative	5	0.013	0.058	4.5
M33	Change	15	23 ($P = 0.0041$)	178.7 ($P = 0.5558$)	7.8 ($P = 0.0407$)
	No change	36	91.7	180.6	2.0
	Negative	5	0	0	
E1 peptide	Change	15	13 ($P = 0.0113$)	44.7 ($P = 0.7881$)	3.4 ($P = 0.0008$)
	No change	36	32.1	37.9	1.2
	Negative	5	3.4	7.2	2.1

[a] *P* values for comparisons between subjects whose sera exhibited a shift in immunoblot pattern from those subjects whose postvaccination sera exhibited no change in RV protein specificities as determined by IgG immunoblot assays.

239 (29, 46), which is one of four known NT domains on this protein (8, 40, 43). GMTs and median antibody levels measured by the three whole-RV EIAs were determined to be well above their established negative cutoff points, suggesting that the majority of the individuals studied had protective levels of RV-specific IgG both before and after receiving the MMR vaccine. Although a clinical correlate of immune protection has not yet been established for antibodies measured by the E1 EIA, a negative cutoff of 10 IU/ml was arbitrarily set to allow for a direct comparison with the other EIA methods. Using this interpretation, seronegativity rates obtained by the E1 EIA were found to be higher than those obtained by the other methods, suggesting that a larger proportion of so-called immune individuals may lack antibodies to this particular NT domain.

Depending on the evaluation method, 2.3 to 3.4% of the 356 subjects followed appeared to be vaccine failures; i.e., they were seronegative before and 1 month after immunization with the MMR vaccine and demonstrated no increase in RV-specific IgG. However, it is possible that these individuals may have been slower responders and, hence, could have become seropositive later. Both the HAI and NT assays are considered to be functional antibody tests and, hence, also evaluate the maturation of the avidity of the specific IgG response, which is time dependent. In contrast, EIAs and immunoblot assays are primary antigen-binding tests and are not as sensitive to differences in antibody avidity. The E1 peptide EIA may represent a hybrid between primary antigen binding and functional antibody tests. This EIA uses as a target antigen a cyclized synthetic peptide, BCH-178c, representing E1 protein residues 213 to 239. Evidence that this sequence contains an NT domain includes the ability of synthetic peptides representing this E1 region (i) to bind to murine monoclonal antibodies with RV-neutralizing activity (8, 29, 43), (ii) to induce NT antibodies in mice and rabbits (13), and (iii) to bind to human sera containing RV NT antibodies (29, 46). In the present study, it was observed that many individuals who would have been considered seropositive by the whole-RV EIAs were actually seronegative (titers, <10 IU/ml) by the E1 peptide EIA. Hence, while they lacked antibody directed to this particular domain, they had antibodies directed to other RV epitopes. In 1978, Schluederberg et al. (38) reported that 10% of sera obtained 3 years after RV HPV77/DE5 vaccination had very low titers of NT antibodies that were also at least fourfold lower than the HAI titers measured in the same samples. Their study also indicated that 10% of RA27/3 vaccine recipients also had low NT titers. These observations signaled potential problems with the duration of protective (NT antibody) immunity in RV vaccinees, particularly those who received the HPV77/DE5 strain which was used before 1979. The subjects in the present study who may have been immunized as children would have likely received the HPV77/DE5 strain as the RV component of the MMR vaccine because the RA27/3 strain (considered more immunogenic than the HPV77/DE5 strain) did not come into widespread use until after 1979. RV reinfection in NT antibody-negative (but HAI antibody-positive) individuals has been the subject of numerous clinical reports (6, 17, 23, 24, 30). Long-term serologic follow-up of RV vaccinees has revealed seroboosting, ostensibly as a result of subclinical wild-type RV reinfection (10) in subjects who were considered to be successfully vaccinated. Moreover, RV challenge studies have revealed that NT antibodies are associated with resistance to reinfection (19). These observations not only indicate the critical importance of RV NT antibodies in immune protection but also reveal potential problems in interpreting RV serologic status by a single method. It is important to note that the domain represented in the synthetic peptide, BCH-178c, is only one of four potential NT domains on the RV E1 protein (8, 40, 43) and that there is evidence for a fifth NT domain on the E2 envelope protein (18). Because these NT domains have been mapped with murine monoclonal antibodies, their importance in immune protection in humans is only inferred. However, the seronegativity rates (17.3% prevaccination and 9.9% postvaccination) that were observed by the E1 peptide EIA in the present study are commensurate with current epidemiologic estimates of protective immunity to RV (7).

By taking advantage of experimental reinfection of individuals with various levels of preexisting RV immunity by reimmunizating them with live attenuated RA27/3 virus, it was possible to examine the influence of preexisting RV antibody on the immune response to the RV vaccine. Because RV antibody screening is routinely performed for adult females of childbearing age, the present study also compared the abilities of different rubella EIA methods to predict protection against accidental RV infection during pregnancy. There is ample evidence that RV infection, either natural or experimental (including challenge infections induced by administration of the live attenuated vaccine strain of RV), is accompanied by a transient viremia, as evidenced by virus shedding from the nasopharynx or blood viremia (4, 16, 33, 37). In many cases, replication of the vaccine strain of virus was associated with a substantial increase (fourfold or greater in the titers of RV-specific IgG and in some instances was accompanied by low levels of RV-specific IgM (3, 33). Our assumption was that an immune response to the parenteral administration of 10^3 infectious virions (the established dose for the RA27/3 vaccine) would not occur without replication of the introduced vaccine strain of RV. Hence, a prevaccination-to-postvaccination change in antibody levels of fourfold or greater was considered to be a surrogate for susceptibility to RV viremia but not necessarily susceptibility to RV disease. It was anticipated that subjects who were initially seropositive would be resistant to RV challenge and vaccine-induced viremia and, consequently, would show no postvaccination increase in RV-specific IgG levels (i.e., the postvaccination/prevaccination ratio would be <2). Instead, as the data in Fig. 1 and 2 and Table 3 suggest, some highly seropositive individuals could be susceptible to reinfection, while others probably are not. Undoubtedly, this reflects individual qualitative differences in the immune response to RV as well as quantitative differences between the detection methods, as evidenced from their relative sensitivities and specificities (Table 2). The M33 and E1 EIAs appeared to be more sensitive in detecting postvaccination increases in antibody levels than the Diamedix and Behring EIAs. This may reflect, in part, differences in the antigen preparations used in the EIAs. The Diamedix and Behring EIAs use intact RV, while the M33 EIA uses detergent-solubilized RV, which likely displays a more diverse array of epitopes (including linear and internal). Hence, the M33 EIA appears to detect a broader range of antibody specificities. This interpretation is supported by the observations of a prevaccination-to-postvaccination shift in the complexity of the RV protein banding pattern (i.e., from E1 only to a combination of E1, E2, and/or C) in the serum samples obtained after vaccination with the MMR vaccine from initially whole-RV EIA-seropositive subjects who were also observed to seroboost by the M33 EIA. This observation also supports the assumption that in these individuals, viral replication had occurred after vaccination, giving rise to additional antibodies directed to newly exposed or internal (C) RV epitopes. Also, more subjects were observed to seroboost by the E1 peptide EIA than by the Diamedix and the Behring EIAs. These individuals all had low E1

peptide antibody levels before vaccination, despite having levels of RV-specific IgG well within the protective limits, as determined by the whole-RV EIAs. Such individuals were also observed to seroboost by the other EIA methods. Similar observations were made by Fogel et al. (16) in intranasal challenge studies with the wild-type (Brown) strain of RV with successfully vaccinated adolescents. In that study fourfold or greater booster responses (as measured by HAI, complement fixation, and NT assays) were observed in 47% of subjects previously vaccinated with the HPV77/DE5 strain. Of those who exhibited booster responses by the HAI and complement fixation assays 65% also had concomitant booster responses in NT assay titers, suggesting that they initially lacked RV-neutralizing antibodies, despite having been vaccinated a few years earlier.

The results of our study suggest that E1 peptide antibody levels of ≥20 IU/ml may protect individuals against RV challenge and subsequent viremia. Is this an advantage? Observations of the natural occurrence of seroboosting (ostensibly as a result of subclinical RV infection) during rubella outbreaks suggest that such periodic natural reimmunizations strengthen both individual and population immunity. On the other hand, do individuals with silent reinfections constitute a population reservoir for RV, and what are the consequences of undetected RV viremia in pregnant women and their unborn children? The risk of fetal damage leading to CRS is considered to be approximately 95% for mothers in whom rubella occurs during the first trimester (15). Anecdotal reports of maternal reinfections with RV which resulted in fetal infection and damage are worrisome, despite conflicting evidence that asymptomatic maternal RV infection leads to fetal damage (11, 25). Whether CRS occurs or not, congenital RV infection is likely to induce immunologic tolerance, resulting in failure to develop adequate postnatal immunity to RV (26a). Thus, on a population basis, the relatively low levels of E1 peptide-specific antibody observed in the present study in subjects who were <21 years of age may be of concern, particularly if they are also found to be low in young women who were vaccinated during childhood. We had observed earlier (29) that, unlike antibodies measured by whole-RV EIAs, E1 peptide-specific antibodies appear to decrease with time after immunization, suggesting selective waning immunity for this NT domain. However, this was not observed in individuals who had been immunized naturally through rubella disease. Also of concern was the relatively larger number of subjects in the present study who, after vaccination with the MMR vaccine, remained seronegative by the E1 EIA. Whether this reflects immunogenetically regulated nonresponsiveness to this region of the RV E1 protein or selective tolerance as a result of perinatal exposure to RV remains to be determined. However, not all subjects who were initially seronegative by the E1 peptide EIA but who were seropositive by the other EIA methods seroboosted. This suggests that antibodies directed to other functional domains on RV proteins may afford protection against RV reinfection. The observation that subjects who had RV E2-specific IgG antibodies before vaccination were less likely to show postvaccination changes suggests a possible role for E2 antibodies in protective immunity.

In conclusion, by their current interpretation levels, none of the EIAs adequately predicted immune protection by our surrogate method. Our observations suggest that certain individuals may be functionally seronegative and may be missed in routine rubella antibody screening programs that use only whole-RV EIAs. Such individuals could be at risk for RV reinfection during disease outbreaks.

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