JOURNAL OF CLINICAL MICROBIOLOGY, July 1992, p. 1841–1847
0095-1137/92/071841-07$02.00/0

Vol. 30, No. 7

# Characterization of Rubella Virus-Specific Antibody Responses by Using a New Synthetic Peptide-Based Enzyme-Linked Immunosorbent Assay

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Received 5 November 1991/Accepted 10 April 1992

**Rubella virus (RV)-specific immunoglobulin G antibodies were studied by enzyme-linked immunosorbent assay (ELISA) techniques in sera from RV (RA 27/3)-vaccinated individuals, patients experiencing natural RV infection, congenital rubella syndrome patients, and individuals failing to respond to repeated RV immunization. Results obtained by using whole-RV ELISAs (detergent-solubilized M33 strain or intact Gilchrist strain) and hemagglutination inhibition (HAI) and neutralization (NT) assays were compared with results obtained with the same sera by using ELISAs employing a synthetic peptide, BCH-178, representing a putative neutralization domain on the RV E1 protein. Murine RV E1-specific monoclonal antibodies with HAI and NT activities exhibited strong reactivity in ELISAs with BCH-178 peptide. In sera from RA 27/3-vaccinated individuals collected at 0 (prevaccine), 1, 2, 3, 4, 5, 6, 12, and 24 to 52 weeks postvaccine, the development of E1-peptide-reactive antibodies closely paralleled increases in RV-specific antibodies measured by whole-RV ELISAs and HAI and NT assays. Similarly, sequential serum samples obtained from patients during acute and convalescent phases of natural RV infection showed a coordinate increase in RV-specific antibodies as measured by whole-RV and peptide ELISAs. Conversely, congenital rubella syndrome patient sera, although exhibiting high levels of antibody in whole-RV ELISAs, had little or no antibody directed to the neutralization domain peptide. Sera from patients failing to respond to repeated RV immunization contained very low levels of RV-specific antibody in all ELISAs. Our results suggest that the sequence represented by BCH-178 peptide may be a previously unidentified neutralization epitope for human antibodies on the RV E1 protein and may prove useful in determining effective RV immunity.**

Rubella virus (RV), a member of the family *Togaviridae* (28), causes a relatively benign, self-limiting illness (German measles) in young children which is usually accompanied by the acquisition of long-lasting immunity (2, 28). However, in women of childbearing age, effective immunity to RV is essential in order to prevent the potentially disastrous consequences of congenital RV infection (28).

RV contains three major structural proteins which have been shown to be antigenic in animals and humans: (i) the two heavily glycosylated envelope proteins, E1 and E2, which protrude as spikes from the virus surface and (ii) the nonglycosylated capsid (C) protein which is closely associated with the viral RNA (7, 20, 26). RV hemagglutinating (HA) activity and at least three domains giving rise to neutralizing antibodies (mapped with murine monoclonal antibodies [MAbs]) have been ascribed to E1 (10, 14, 22, 27). While the function of E2 is largely unknown, at least one weak neutralization domain has been identified on the protein with MAbs (10). Although it is assumed that the neutralization domains recognized by mouse MAbs are the same as those recognized by human antibodies, this has not been established definitively. Also unknown are the epitopes bound by antibodies directed to E2 and C and what their roles are.

RV-specific immunity in both adults and children is usually assessed by measuring RV-specific immunoglobulin G (IgG) or IgM antibodies in serum. Originally, hemagglutination inhibition (HAI) and neutralization (NT) methods were used, as these were shown to correlate well with the development of protective immunity (17). However, as these assays were either difficult to perform or difficult to standardize, they have, over the years, been replaced by more facile, rapid, and reproducible enzyme-linked immunosorbent assays (ELISAs). Although most ELISAs in current clinical use have been validated against the "gold standard" HAI or NT methodology, solid-phase assays such as the ELISA have the potential to detect all antigenically reactive antibodies, including those which may not play a protective role. Also, certain individuals may fail to produce antibodies directed to protective epitopes, such as the neutralizing domains of E1 and E2, yet produce antibodies directed to other antigenic subregions of RV proteins. ELISAs employing whole RV would not distinguish between the various antibody specificities. For example, women who were deemed to be seronegative by HAI titers of ≤1:8 were shown to have moderate levels of RV-specific antibody measurable by ELISA employing whole detergent-solubilized RV (25). Similar observations with sera obtained from congenital rubella syndrome (CRS) patients have been made (unpublished data). Reexamination of these sera by immunoblot assay has often shown a preponderance of antibody directed to E2 with low or absent reactivity with E1. Hence, seroconversion as measured by whole-RV ELISA techniques may not always correlate with protection from disease, especially if there has been a failure to recognize and to produce antibodies directed to neutralizing epitopes. Failure to produce neutralizing antibodies may result in viral persis-

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TABLE 1. Patient study groups

| Study group (sex[a]) | Age ($\bar{x}$ ± SEM)[b] | No. of patients | Characteristics of serum samples |
|---|---|---|---|
| RV (RA 27/3) | | | |
| F | 23.3 ± 0.6 | 44 | 0, 1, 2, 3, 4, 5, 6, 12, and 24+ weeks postvaccine |
| Natural RV infection | | | |
| F | 24.8 ± 1.1 | 67 | 0, 0.5, 6, and 18+ months postonset of rubelliform rash |
| M | 16.9 ± 1.1 | 32 | |
| CRS | | | |
| F | 11.4 ± 1.2 | 47 | Randomly selected samples taken at various intervals |
| M | 10.9 ± 1.4 | 36 | |
| Failed rubella immunization | | | |
| F | 25.9 ± 1.7 | 27 | Randomly selected samples taken at various intervals 1 to 2 years postrubella immunization, HAI titers of <1:8 |
| M | 20 | 1 | |

[a] F, females; M, males.
[b] Age at time serum sample was obtained.

tence and/or chronic antigenemia with production of immune complex-associated pathology (4, 5, 24). We have previously reported RV persistence in the peripheral blood and synovial fluid of certain individuals with RV-associated arthritis following natural infection or immunization (4, 23), and RV has been isolated from the same tissues in certain children with juvenile rheumatoid arthritis (5). Therefore, the specificity and avidity of antibodies generated in the immune response to RV for individual viral proteins or their antigenic subregions may be more critical than their quantity. Current (first-generation) ELISA methodology utilizes whole RV as a target antigen for detecting specific antibodies. Although it is assumed that these tests detect reactivity with all RV epitopes, this may not be the case. Second-generation tests such as immunoblots or ELISAs employing purified or recombinant RV proteins as target antigens will better define the specificities of the antibodies generated in response to RV. Third-generation ELISAs will utilize antigenic subregions of RV proteins represented by synthetic peptides once the relevant epitopes have been identified for the human antibody response. In the study reported herein, we have compared the antibody-response profiles of individuals exposed to RV (by natural infection or immunization) by using ELISAs employing intact or detergent-solubilized whole RV and an ELISA employing a synthetic peptide representing a linear sequence of E1. Using clinically documented sequential serum samples obtained from congenitally or postnatally RV-infected or immunized individuals, we have determined typical antibody-response profiles as assessed by whole-RV and synthetic-peptide ELISAs and HAI and NT antibody assays. We show here that the sequence represented by the E1 peptide, BCH-178, although different from those reported to be involved as neutralization domains by other investigators (18, 22), may represent a target for neutralizing antibodies in the human response to RV.

## MATERIALS AND METHODS

**Patient samples.** During the past 10 years, individual serum samples have been collected at various time intervals relative to natural RV infection or rubella immunization from healthy adults and children experiencing natural RV infection, adult females prospectively studied after immunization with rubella vaccine (RA 27/3 strain Meruvax; Merck Sharp & Dohme), individuals congenitally infected with RV (CRS), and individuals exhibiting repeated failure to seroconvert (by HAI or ELISA techniques) to RV infection or immunization. Table 1 summarizes these patient study groups. In the majority of cases monitored, sequential serum samples were collected at known intervals after infection or immunization and the clinical outcomes of RV exposure were documented. All serum samples were stored in small aliquots at −70°C and thawed just before use.

**RV-specific MAbs.** RV-specific murine MAbs were a generous gift from Shirley Gillam (Department of Pathology, University of British Columbia, Vancouver, Canada). The panel of MAbs used had been partially characterized and were shown to immunoprecipitate RV-E1 protein and to exhibit HAI and/or NT antibody activities (1).

**RV preparations.** RV M33 strain (RV: ADCC, VR-315) was grown on Vero cell monolayers and harvested from tissue culture fluids. Supernatants were initially clarified by centrifugation at 1,000 × *g* for 15 min at room temperature. For each virus preparation, virus titers (in PFU) were determined by plaque formation on RK13 cell monolayers (11). RV in supernatants was concentrated either by polyethylene glycol (10% [wt/vol]) precipitation or by ultracentrifugation at 27,000 × *g* for 2 h at 4°C. For ELISAs, the resulting viral pellets were resuspended in phosphate-buffered saline (PBS) (150 mM NaCl, 60 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 2 mM KCl, 0.02% [wt/vol] $NaN_3$) containing 0.5% Triton X-100.

**BCH-178 synthetic peptide.** BCH-178 is a synthetic peptide developed by IAF BioChem International, Inc., which covers amino acid residues 213 to 239 of the E1 protein of RV (Fig. 1). This peptide was prepared by automated solid-phase synthesis, its constitutive cysteine residues were linked through a disulfide bridge by using conventional methods (8, 19), and it was purified by reverse-phase high-pressure liquid chromatography before being coated onto ELISA plates. The sequence of BCH-178 lies in a conserved region of E1 and is distal on the amino terminus of E1 to the previously described EP1, -2, and -3 epitopes (10, 14, 18, 22).

**Whole-RV ELISAs.** ELISAs to detect IgG antibodies binding to RV antigens were performed as previously described (24). Detergent-solubilized whole RV (M33 strain) prepared as described above was optimally diluted in carbonate-bicarbonate buffer (pH 9.8) and coated onto enzyme immunoassay plates (Falcon 3915) by overnight incubation at 4°C. After unbound RV antigen was discarded and the plates were washed with PBS containing 0.05% Tween 20 and 0.05% (wt/vol) bovine serum albumin (BSA) (PBST), the plates were blocked for 1 h at room temperature with PBS containing 0.5% (wt/vol) BSA and then washed three times


FIG. 1. Amino acid sequence of the RV E1 glycoprotein. The amino acid sequence of the RV E1 envelope protein (Judith strain) is depicted. The positions of the EP1, EP2, and EP3 epitopes previously defined with MAbs as HAI and neutralization domains are indicated with a thin underline. The relative position of the BCH-178 peptide is indicated by a thick underline.

in PBST and flicked dry. Next, undiluted tissue culture supernatants containing MAbs or human serum serially diluted in PBS-BSA were added in duplicate 100-μl aliquots to antigen-coated wells and incubated at 37°C for 1 h and then were washed four times in PBST. In assays for RV-specific human IgG, each plate contained a five-point dilution series of pooled reference sera which was standardized against the WHO International RV-IgG reference serum (30) and was shown to contain 943 IU/ml before dilution. To detect specific IgG antibodies, optimally diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins (DAKO Corporation, Santa Barbara, Calif.) or alkaline phosphatase (AP)-conjugated goat anti-human IgG (γ) (affinity purified, from Kirkegaard & Perry Laboratories, Gaithersburg, Md.) were added for a further 1-h incubation at 37°C. After the unreacted enzyme conjugates were discarded and the plates were washed in PBST, enzyme substrate, tetramethylbenzidine-hydrogen peroxide (TMB-$H_2O_2$) (Sigma Chemical Co., St. Louis, Mo.) for HRP conjugates or *p*-nitrophenylphosphate (2.5 mg/ml in diethanolamine-$Mg^{2+}$ buffer [pH 9.8]) for AP conjugates, was added. The plates were incubated at 37°C until the net absorbance of the most concentrated reference standard wells was determined to be 1.000 U. The enzyme reaction was stopped by adding 50 μl of 1 M NaOH (AP conjugates) or 1 M HCl (HRP conjugates) to each well, and the absorbances for each well were determined by using a Bio-Rad model 3550 microplate reader. Concentrations of RV-specific IgG (in IU/ml) were determined by linear regression analysis from standard curves prepared for each assay plate.

Human sera were also tested for RV-specific IgG antibodies by a clinically validated commercial assay (Rubella IgG Microassay; DiaMedix Corporation, Miami, Fla.) which employs intact RV (Gilchrist strain). All sera were tested at a single dilution (1:41), and the assay was performed according to the manufacturer's instructions. Results were expressed as ELISA units/ml after the calculation of absorbance ratios relative to a reference serum (calibrated against the WHO International RV-IgG reference) provided with the assay kit.

**BCH-178 synthetic-peptide ELISA.** BCH-178, an RV-E1 synthetic peptide (IAF BioChem International, Inc.), was coated onto enzyme immunoassay microplates (Immulon 4; Dynatech, Chantilly, Va.) at a concentration of 10 μg/ml in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were air dried and stored in hermetically sealed aluminum foil bags at 4°C until required. Human sera were diluted to 1:51 by using the sample dilution buffer provided with the manufacturer's kit. Murine MAbs were added to the peptide antigen-coated wells as undiluted tissue culture supernatants. Each assay included positive and negative prediluted control serum samples as well as low-level RV-IgG calibrator serum (15 IU/ml) provided by the manufacturer. IgG antibodies were detected with optimally diluted HRP-conjugated goat anti-human IgG (γ) provided by the manufacturer. All incubations were performed at room temperature for 30 min. Wells were washed five times with PBST after each antibody incubation. After the last incubation, enzyme substrate (TMB-$H_2O_2$) was added to the wells and incubated for 30 min at room temperature. The enzymatic reaction was stopped by adding 100 μl of 1 M HCl to each well, and the $A_{450}$ was determined as described above. Results were expressed as IU/ml after the calculation of absorbance ratios relative to the calibrator included on each plate.

**Rubella HAI and NT antibody assays.** HAI and NT antibody assays for RV-specific IgG (after the separation of IgM by density gradient centrifugation) were performed on selected serum samples by the Provincial Virology Laboratory, Vancouver, British Columbia, Canada, by using established methods.

**Statistical methods.** Sample data were compared by using Student's *t* test for unpaired samples and/or the Mann-Whitney U test after preliminary examination of natural log-transformed data. Relationships between patient age or time after exposure to RV and test results were determined by linear regression analysis and by examination of scatter plots.

## RESULTS

**Reactivity of MAbs with whole RV and BCH-178 synthetic peptide.** Preliminary examination in one of our laboratories (IAF BioChem) of pre- and post-RV immunization serum samples from RA 27/3-vaccinated females (data not shown) revealed that the development of RV-specific IgG antibodies as measured by a commercial ELISA (Behring Enzygnost; Berhringwerk AG, Marburg, Germany) and BCH-178 peptide paralleled the development of RV HAI antibodies in these serum samples. In order to further examine the relationship between E1 peptide ELISA reactivity and functional antibody activity as measured by HAI and NT antibody assays, we determined the reactivity of a panel of E1-specific murine MAbs with whole RV (detergent-solubilized M33 strain) and BCH-178 synthetic peptide in ELISAs. These MAbs had been partially characterized in the laboratory of S. Gillam (Department of Pathology, University of British Columbia) and were shown to immunoprecipitate E1 protein from both M33 and RA 27/3 strains of RV and to have various amounts of HAI and NT antibody activity (Table 2). All MAbs also showed good reactivity with whole RV and BCH-178 peptide (Table 2).

**Development of RV-specific antibodies following immunization with RA 27/3 vaccine.** RV-specific IgG antibody responses in a group of 44 female university students who had initially been determined to be seronegative by HAI (titers of ≤1:8) were studied. Serum samples were obtained from each vaccinee just prior to (0) and at 1, 2, 3, 4, 5, 6, 12, and 24 weeks after a single dose of RA 27/3 vaccine. RV-specific IgG antibodies were measured by two different ELISAs

TABLE 2. Reactivity of RV-specific MAbs with RV and the RV-E1 peptide, BCH-178

| MAb[a] | ELISA absorbance units | | Reciprocal titer | |
|---|---|---|---|---|
| | RV | BCH-178 | HAI[b] | NT[c] |
| 12B2D | 0.807 | 2.840 | 4096 | 20 |
| 12B3G | 1.497 | 2.821 | <8 | 5 |
| 12B6E | 1.484 | >3.000 | ND[d] | ND |
| 13A4H | 0.882 | 0.236 | <8 | 5 |
| 21B6A | 1.862 | >3.000 | ND | ND |
| 21B9H | 2.010 | 2.716 | <8 | 20 |
| 2F10G | 1.618 | 2.792 | ND | ND |
| 16A10E | 1.399 | 2.829 | 32 | 5 |
| 21B6B | 2.002 | >3.000 | ND | ND |

[a] All MAbs are specific for E1 as determined by immune precipitation (1).
[b] HAI test (1).
[c] Neutralizing antibody assay (1).
[d] ND, not determined.

which employ whole RV, an in-house ELISA with detergent-solubilized M33 strain (Fig. 2A) and the DiaMedix Rubella IgG Microassay, which uses intact Gilchrist strain virus (Fig. 2B), as well as by an ELISA with BCH-178 peptide (Fig. 2C). RV-specific IgG antibody levels measured by the BCH-178 peptide ELISA were also compared with levels of antibody measured by HAI and NT antibody assays in sera from vaccinated individuals (Fig. 3). It was observed that the development of RV-specific IgG antibodies measurable by the BCH-178 peptide ELISA closely paralleled that of those measured by the other assay methods. In fact, all but one vaccinee had seroconverted by the synthetic peptide ELISA by week 4. Regression analysis of the results obtained from each serum series of each vaccinee revealed good correlations between methods. The $\bar{x}$ values ± standard errors of the mean (SEM) of the regression coefficient ($r$) for the following methods were as follows: DiaMedix RV ELISA, 0.7863 ± 0.0202; whole-RV (M33) ELISA, 0.6550 ± 0.0281; HAI antibody assay, 0.7313 ± 0.0349; and NT antibody assay, 0.7319 ± 0.0276.

**Development of RV-specific IgG following natural RV infection.** The development of RV-specific IgG antibodies following natural RV infection in 99 male and female patients was studied (Table 1). Three time intervals were chosen for study: (i) early acute phase (0 to 6 days postonset of rubelliform rash), (ii) acute phase (8 to 28 days postonset), and (iii) convalescent phase (1 to 36 months postonset). Comparison of RV-specific antibody responses in three sequential serum samples taken from each patient measured during these time intervals with specific responses in vaccinated individuals measured at comparable time intervals is shown in Fig. 2. Specific IgG responses were determined by using the two whole-RV ELISA methods (Fig. 2A and B) and the BCH-178 peptide ELISA (Fig. 2C) as described above. Within each interval, linear regression analysis and scatter plots indicated no significant relationships between patient age, the time since the onset of rash, and antibody values measured in these assays (data not shown). However, significant differences ($P \leq 0.022$) in mean IgG antibody levels were observed between intervals. As was observed for the samples obtained from RV-vaccinated individuals, the development of RV-specific IgG antibodies in naturally infected patients as measured by the whole-RV ELISAs closely paralleled the increases in antibody levels reactive with BCH-178 peptide.

In general, levels of RV-specific IgG were observed to be higher in serum samples obtained from naturally infected patients than those from vaccinated individuals (Fig. 2).

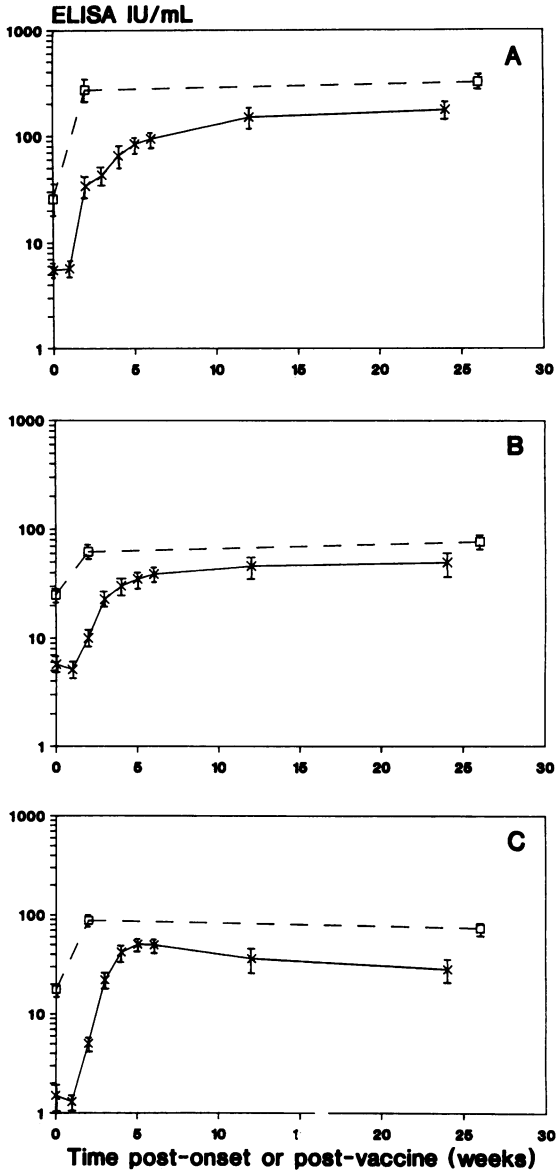


FIG. 2. Comparison of IgG antibody levels measured by ELISA in vaccinated and naturally infected individuals. Sequential serum samples were collected from RV vaccinees (×) at 0 (prevaccine), 1, 2, 3, 4, 5, 6, 12, and 24 to 52 weeks postvaccine and RV-infected patients (□) during the early acute phase (0 ± 1.2 weeks, $\bar{x}$ ± SEM, postonset of rubelliform rash), the acute phase (1.8 ± 0.1 weeks, $\bar{x}$ ± SEM, postonset), and the convalescent phase (25.5 ± 2.8 weeks, $\bar{x}$ ± SEM, postonset) of infection. RV-specific IgG antibodies in these sera were measured by whole-RV ELISA using the detergent-solubilized RV-M33 strain (A), the DiaMedix Rubella IgG Microassay (B), and the BCH-178 (RV-E1) peptide ELISA (C). Results shown are the arithmetic mean ± SEM of determinations from all serum samples obtained from patients in each group at the time points indicated.

**RV-specific antibody levels in CRS patients.** Randomly selected serum samples from 52 CRS patients were tested for RV-specific IgG antibodies by using whole-RV ELISAs (in-house and DiaMedix assays) and the BCH-178 peptide

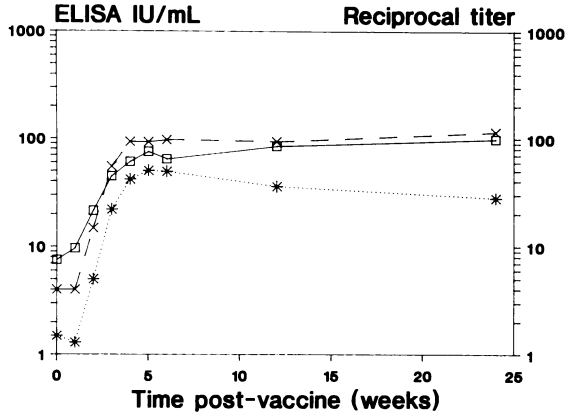


FIG. 3. Development of RV-E1 peptide-reactive, HAI, and neutralizing antibodies after RV vaccination. Sequential serum samples were collected from adult females ($n$ = 44) at 0 (prevaccine), 1, 2, 3, 4, 5, 6, 12, and 24 to 52 weeks after a single dose of RA 27/3 vaccine. RV-specific IgG antibody levels in these sera were measured by BCH-178 (E1) peptide ELISA (*) and HAI (×) and neutralizing antibody (□) assays. Results shown represent the arithmetic mean of determinations from all serum samples obtained at each time point. Error bars have been omitted for clarity.

ELISA. Comparative results of these assays performed with each group of serum samples are shown in Fig. 4. Levels of specific IgG antibody measured by the in-house whole-RV ELISA and the DiaMedix assay were 200.0 ± 2.7 and 56.3 ± 1.1 IU/ml ($\bar{x}$ ± SEM), respectively, and therefore were well above the established protective level of 15 IU/ml. However, levels of antibody measured by the BCH-178 peptide ELISA were very low in the majority of the CRS samples tested (10.0 ± 1.1 IU/ml, $\bar{x}$ ± SEM) in comparison with the levels observed in late-phase samples (convalescent phase and week 24+) from healthy naturally infected (73.1 ± 3.1, $\bar{x}$ ± SEM) and vaccinated (28.1 ± 3.4, $\bar{x}$ ± SEM) individuals.

**RV-specific antibody levels in individuals demonstrating failed rubella immunization.** RV-specific IgG antibody levels in randomly selected serum samples ($n$ = 21) from individuals who had demonstrated failure to seroconvert (by HAI assay or other commercial ELISA techniques employed in the Provincial Virology Laboratory) after repeated exposure to RV by vaccination or by natural infection were determined. Levels of IgG antibody measured in individual serum samples by the whole-RV ELISAs (in-house and DiaMedix assays) and by the BCH-178 peptide ELISA are shown in Fig. 5. Levels of specific IgG antibody in these sera as measured by the in-house whole-RV ELISA (19.0 ± 3.7 IU/ml, $\bar{x}$ ± SEM) and the DiaMedix ELISA (15.3 ± 2.4 IU/ml, $\bar{x}$ ± SEM) were low, barely above established protective levels. Levels of IgG antibody measured by the BCH-178 peptide ELISA were also very low (5.3 ± 0.8 IU/ml, $\bar{x}$ ± SEM) in comparison with levels measured in healthy immunized or naturally infected individuals.

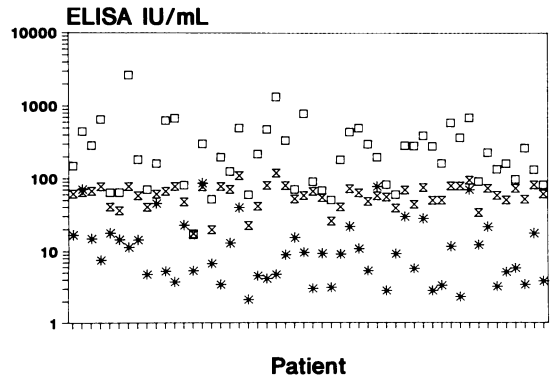


FIG. 4. RV-specific IgG antibody levels in CRS patients. Randomly selected serum samples obtained from CRS patients were tested for RV-specific IgG antibodies by whole-RV ELISA using detergent-solubilized RV-M33 strain (□), the DiaMedix Rubella Microassay ( ⧖ ), and the BCH-178 (E1) peptide ELISA (*).

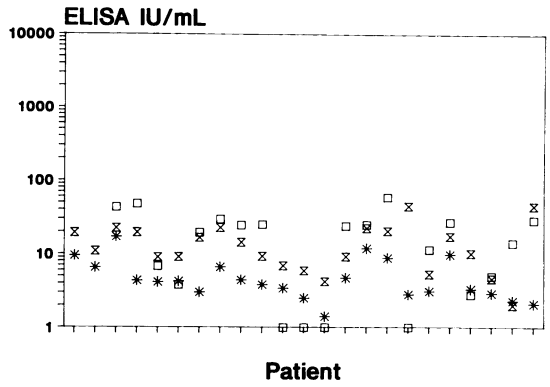


FIG. 5. RV-specific IgG antibody levels in individuals exhibiting failed rubella immunization. Randomly selected serum samples obtained from individuals showing failure to seroconvert to RV immunization (by HAI or commercial ELISA techniques) were retested for RV-specific antibodies by whole-RV ELISA using detergent-solubilized RV-M33 strain (□), the DiaMedix Rubella IgG Microassay ( ⧖ ), and the BCH-178 (E1) peptide ELISA (*).

## DISCUSSION

The observed increase of IgG antibody levels reactive with the synthetic peptide BCH-178, in parallel with the development of antibodies measured by whole-virus ELISA techniques and functional assays (HAI and NT), strongly suggests that this epitope, which is located on the E1 protein of RV, is closely linked with the development of protective immunity to RV. This synthetic peptide is also recognized by a series of murine MAbs which are specific to E1 (as determined by immunoprecipitation) and capable of neutralizing RV in culture. The amino acid sequence reproduced by BCH-178 does not overlap the EP1, EP2, and EP3 hemagglutinating and neutralizing domains of E1 defined by Terry et al. (22) with murine MAbs. However, the BCH-178 sequence is adjacent to the EP2 epitope, which was shown by these investigators to have HA activity and to react with RV-neutralizing MAbs. The BCH-178 sequence also falls within the E1 region recently shown by Wolinsky et al. (29) to be recognized by murine MAbs with both RV NT and HAI activities. In prospectively studied rubella vaccinees, IgG antibody levels reactive with BCH-178 peptide in ELISA were observed to increase in parallel with those measured by two whole-RV ELISA techniques (one of which, the DiaMedix ELISA, has been validated for clinical use) and two functional antibody tests (HAI and NT). The high degree of correlation observed between the values obtained by the different assay methods suggests that the

BCH-178 peptide assay likely has good predictive capacity for evaluating effective immunity to RV in a vaccinated population. As measured by the peptide ELISA, the levels of antibody necessary to confer protection remain to be defined; however, our data suggest that the cutoff level of 15 IU/ml used for the current whole-RV ELISA methods may also be suitable for the BCH-178 peptide ELISA. In the RA 27/3-vaccinated individuals whose data are shown in this study, levels of antibody measured by the BCH-178 peptide ELISA were observed to drop off after 24 to 52 weeks postvaccine, so that only 71.4% of the group showed antibody levels of ≥15 IU/ml ($\bar{x}$ ± SEM = 28.1 ± 3.4 IU/ml), compared with 90.9% ($\bar{x}$ ± SEM = 49.3 ± 4.2 IU/ml) at 6 weeks postvaccine. Whether this represents a time-related loss of antibody reactive with this E1 region in all vaccinated individuals or a unique feature of this patient study group remains to be determined. However, the adult females in this vaccine group may be atypically seronegative individuals who have lost their previously established immunity to RV, whereas a significant proportion of the general population might be expected to show some degree of RV seropositivity due to previous vaccination and/or natural infection. This question is currently being addressed in studies involving unselected vaccinees and will be the subject of a future manuscript.

BCH-178 peptide-reactive IgG antibody levels in normal individuals responding to natural RV infection or to immunization with a single dose of RA 27/3 vaccine were compared. In the naturally infected patient group, these antibodies peaked during the acute phase (8 to 30 days postonset of rash) and were observed to decline somewhat in convalescent-phase sera. Levels of antibody observed in the naturally infected group were generally higher than the mean levels of RV-specific antibody measured in the vaccine group (Fig. 2). This has also been observed by others in studies of the cell-mediated immune response to RV (2). In both groups, levels of RV-specific antibody measured by the peptide ELISA quantitatively paralleled those measured by the DiaMedix whole-RV ELISA, which employs intact Gilchrist strain virus as an antigen. In contrast, levels of IgG antibody measured by our in-house whole-RV ELISA, which uses detergent-solubilized M33 strain virus, were quantitatively similar to those measured by the DiaMedix and peptide ELISAs in early sera but were considerably higher in later sera. Although this may have reflected strain differences in the RV preparations (7), it is more likely that the observed dissociation is due to more diverse specificities in late-phase sera and to the ability of these antibodies to react with internally located C epitopes, or those otherwise exposed on E2 in the detergent-solubilized virus preparation, in addition to E1. It is also likely that the predominant antigen presented by the intact RV used in the DiaMedix test is E1 protein. The observation of E2 and C antibodies in parallel immunoblots performed with late-phase sera, in contrast to early-phase sera, which show predominantly E1 patterns of reactivity (data not shown), lends credibility to this interpretation.

Our observation of very low levels of antibody reactive with BCH-178 peptide in CRS patients and in patients who have exhibited repeated failure to respond to RV immunization lends further credence to the theory that the E1 region represented by this peptide may itself be a significant target for protective antibodies. Antibodies directed to the BCH-178 domain may function by sterically inhibiting the attachment of RV to HA receptors on erythrocytes or membrane receptors on host cells that are targets for viral replication. Alternatively, this domain, as an adjacent epitope to the previously established neutralization and HAI domains, may also be recognized antigenically in the context of an effective immune response to RV. It is well established that congenital RV infection may be associated with long-term virus persistence in tissues and excretion of RV postnatally, as well as abnormal patterns of RV immunoreactivity (3, 6, 12, 16). RV persistence in CRS patients may be due to development of selective immunologic tolerance to neutralization domains on RV E1 protein as a result of exposure of the immature fetal immune system to RV. The extremely low levels of antibodies reactive with the domain represented by BCH-178 peptide observed in CRS patients suggest that this indeed may be the case. Similarly, lack of responsiveness to biologically relevant regions of RV E1 protein may lead to failed immunity to RV and reinfection. Numerous cases concerning the lack of association of immunity as determined by gold standard techniques such as HAI and neutralizing antibody tests and RV reinfection have been documented (9, 12, 15, 21). This is of considerable concern because of the risk of RV reinfection in pregnant women (9, 13).

ELISA techniques employing whole RV have permitted more facile and reliable serodiagnosis of rubella immunity. However, it is becoming increasingly clear that specific immunity should be assessed on a functional-epitope basis. This will also be important in the design of future vaccines which will address the influence of immunogenetic background on the development of effective immunity to RV. The availability of rapid, simple, and reproducible ELISAs employing synthetic peptides representing biologically relevant areas of RV proteins will greatly facilitate this process.

## ACKNOWLEDGMENTS

This work was supported by research grant MT-7351 (Medical Research Council of Canada). Aubrey Tingle is a recipient of the Career Investigatorship Award of the B.C. Children's Hospital Foundation.

We gratefully acknowledge Murray MacKinnon for statistical advice and Jennifer Allan and Deborah Brown for expert assistance in preparing the manuscript.

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