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Analysis of T- and B-Cell Epitopes of Capsid Protein of Rubella Virus by Using Synthetic Peptides

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A nested set of 11 overlapping synthetic peptides covering the entire sequence of rubella virus capsid protein was synthesized, purified, and tested against human rubella virus-specific T-cell lines and rubella virus-seropositive sera. T-cell lines derived from four donors responded strongly to four synthetic peptides containing residues 96 to 123, 119 to 152, 205 to 233, and 255 to 280. Only one peptide (residues 255 to 280) was recognized by all four T-cell lines. Two human immunodominant linear B-cell epitopes were mapped to residues 1 to 30 and 96 to 123 by using peptide-specific enzyme-linked immunosorbent assay. All 11 synthetic peptides were highly immunogenic and induced strong antibody responses in rabbits against the respective immunized peptides. Seven of the 11 rabbit antipeptide antisera (anti-1–30, -74–100, -96–123, -119–152, -205–233, -231–257, and -255–280) specifically recognized the capsid protein on immunoblots. Identification of these T- and B-cell epitopes represents the first step toward rational design of synthetic vaccines against rubella.

Rubella is usually a benign childhood infection, but rubella virus (RV) can cause a persistent infection of the brain called progressive rubella panencephalitis (53). RV has been isolated from peripheral blood lymphocytes of women with rubella-associated arthritis (7) and from synovial cells of patients with juvenile rheumatoid arthritis (15). Minor antigenic differences have been observed between wild-type and vaccine strains of RV (22). These differences may be responsible for the high rate of reinfections in individuals with vaccine-induced rather than natural immunity to rubella (33, 49). In addition to these problems, RV grows to relatively low titer and its structural proteins are difficult to purify. Therefore, it is clear that a new approach is required to design a safer and effective rubella vaccine, especially for susceptible pregnant women. Efforts have recently focused on characterizing both the viral genome and the host immune responses.

RV is the sole member of the genus *Rubivirus* in the family *Togaviridae* (30). The primary sequences of the RV structural proteins decoded from the cDNA clones have been reported (9). The RV virion contains an RNA genome enclosed within an icosahedral capsid composed of multiple copies of a basic capsid (C) protein of 33 kDa (39). Surrounding this nucleocapsid is a lipid bilayer in which viral glycoproteins E1 (58 kDa) and E2 (42 to 47 kDa) are embedded (39). Glycoprotein E1 has been shown to contain hemagglutinin and virus neutralization epitopes (52). E2-specific antibodies are capable of neutralizing viral infection in vitro (18). Studies have been carried out to characterize the specificity of the antibody response against rubella. A rubella-specific immunoglobulin M (IgM) response is widely used for diagnosis of recent RV infection (20), and the production of rubella-specific IgA antibodies has been shown to be important in the prevention of reinfection (20). Much of the rubella-specific IgM reacts with the E1 protein, while most of the IgA reacts with the C protein (43). IgG antibody response can be elicited by all of the structural proteins (43).

Little is known about the cellular immune response to RV structural proteins, although both T-helper cell proliferation (3, 24, 29, 51) and cytolytic T-lymphocyte responses (48) can be measured during viral infections. An understanding of the humoral and cellular immune responses may lead to development of a safe and effective rubella vaccine. In this study, we attempt to define human T- and B-cell epitopes of the C protein of RV, using 11 overlapping synthetic peptides covering its entire sequence (9).

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MATERIALS AND METHODS

Virus. RV strain M33 (ATCC VR-315) was grown in Vero cells and isolated from the culture supernatant as described previously (9). The virus pellets were suspended in a small volume of phosphate-buffered saline (PBS) and stored at −70°C. The virus stock was titrated in RK 13 cells, using an immunocytochemical focus assay modified from methods described elsewhere (16, 40, 52). The virus stock (5×10^7 focus-forming units/ml) was inactivated by UV light (254 nm; model UVG-54; UV Products Inc.) for 10 min before use.

Peptide synthesis. Peptides were synthesized in an automated ABI 430A peptide synthesizer, using solid-phase methods (32). Eleven peptides (27 to 34 residues in length) covering the entire sequence of RV C protein were synthesized. In some peptides, an additional cysteine residue was added at the C terminus for coupling to a carrier. Synthetic peptides were cleaved from the resin by treatment with hydrogen fluoride and purified by reverse-phase high-pressure liquid chromatography using a Vydac C4 column. The purity of all peptide preparations exceeded 95%. For each peptide, amino acid analyses were performed on a Waters Pico-Tag system and found to be in good agreement with the theoretical composition.

Rabbit immunization. To prepare peptide-specific antisera, two New Zealand White rabbits (Maple Lane Farm, Ontario, Canada) were immunized intramuscularly with 100 μg of peptide emulsified in Freund's complete adjuvant. On days 14 and 28, each rabbit received booster injections with the same immunogens in incomplete Freund's adjuvant. Rabbit sera were collected 2 weeks after the final booster injection, heat inactivated at 56°C, and then stored at −20°C.

RV-specific T cells. Peripheral blood mononuclear cells (PBMC) from RV-seropositive humans (AT, RM, CM, DO, MS, and TZ) were isolated by centrifuging the heparinized blood on a Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc.) gradient for in vitro stimulation as previously described (4, 6). PBMC (2.5×10^6 cells per ml) in a 24-well plate (GIBCO) were incubated with UV-inactivated RV (5×10^5 focus-forming units/ml) in a complete medium (RPMI 1640 [Sigma] containing 2 mM L-glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 50 mM penicillin, 50 mM streptomycin, and 5×10^{-5} M 2-mercaptoethanol) supplemented with 10% autologous plasma. After incubation at 37°C for 7 days, the cells were washed three times with medium and resuspended at 10^6 cells per ml in a complete medium supplemented with 10% fetal calf serum and 100 U of human recombinant interleukin-2 (Cetus) per ml. After 7 days of incubation, an antigen-induced proliferative assay was performed.

Proliferation assay. RV-stimulated T lymphocytes (2×10^4 cells per well) were incubated with autologous, γ-irradiated (3,000 rads) PBMC (5×10^4 cells per well) in a complete medium containing 10% fetal calf serum and different concentrations of antigen (individual capsid peptides or inactivated RV) in triplicate in 96-well round-bottom plate for 3 days as previously described (5, 6). The cell cultures were pulsed with 1 μCi of [^{3}H]thymidine (DuPont) per well for the last 15 to 20 h. After the cells were harvested with a cell harvester (Cambridge Technology PHD), incorporated radioactivity was determined in a liquid scintillation counter. Results are presented either as mean counts per minute of three replicate determinations with the standard error of the mean or as the cell proliferative index, which is the ratio of the mean counts per minute incorporated in the presence of antigen to the mean counts per minute obtained in the absence of antigen (background). A cell proliferative index of 2 or more was considered significant.

Determination of cell surface antigens. T cells (2×10^5) cultured for a short time were incubated with phycoerythrin-labeled murine monoclonal antibody CD3 (IOT3-PHYCO), CD4 (IOT4-PHYCO), or CD8 (IOT8-PHYCO) (AMAC Inc.) or normal mouse serum for 0.5 h on ice (4). The cells were washed three times with PBS containing 5% fetal calf serum and then subjected to cytometric analysis in a fluorescence-activated cell sorter (FACS) (EPICS; Coulter Electronic Co.). Pooled normal BALB/c mouse serum was used as a negative control.

Peptide-specific ELISA. Microtiter plates (Nunc-Immuno; Nunc, Roskilde, Denmark) were coated with peptide (1 μg per well) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) overnight at room temperature as previously described (17, 47). The wells were blocked with diluent (0.5% bovine serum albumin [BSA]–0.5% Tween 20 in PBS) for 1 h, and serum samples (human, rabbit, or mouse antisera) were added at dilutions from 1:16 to 1:2,048 for 1 h. The wells were washed five times with washing buffer (PBS containing 0.1% BSA and 0.5% Tween 20), and affinity-purified phosphatase-conjugated goat antibody to human IgG, to mouse IgG, or to rabbit IgG (Kirkegaard and Perry Laboratories) was added. After 1 h of incubation, the plates were washed five times and then developed by addition of *p*-nitrophenyl phosphate (2.5 mg/ml) in 10% diethanolamine–0.01% $MgCl_2$ (pH 9.8) at 160 μl per well. After 0.5 h, the optical density was measured at 405 nm (model 3550 microplate reader; Bio-Rad Laboratories, Richmond, Calif.). Human RV-negative serum and preimmunized rabbit or mouse serum were used as negative controls. Seropositivity for human serum was defined as any value greater than the mean of the negative control plus 3 standard deviations (mean + 3 SD) scored as +, values more than twofold greater (negative control mean + 3 SD) scored as ++, and values more than threefold greater (negative control mean + 3 SD) scored as +++. Negative control values for the human peptide enzyme-linked immunosorbent assay (ELISA) were established by running each peptide with a panel of negative control sera and averaging the absorbance value. Each specimen was tested at least three times.

Immunoblot analysis. Purified RV particles were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels containing 0.1% SDS. After electrophoretic separation, proteins were transferred to cellulose nitrate membranes (Hybond C; Amersham). Membranes were blocked in Tris-buffered saline (0.15 M NaCl, 0.02 M Tris-Cl [pH 7.5]) containing 4% powdered skimmed milk and incubated with either human anti-RV serum (1:80 dilution) or rabbit anti-C-peptide serum (1:100 dilution). The proteins were visualized by using peroxidase-conjugated anti-human IgG or anti-rabbit IgG (Dako Corp.). Densitometric tracings on the immunoblot strips were made by using a video densitometer (model 620; Bio-Rad), and the relative density of bands was determined by the integration of the area of absorbance peaks.

RESULTS

Selection of peptides. To map the T- and B-cell immunodominant epitopes of RV C protein as a basis for the rational design of a synthetic peptide-based rubella vaccine, we synthesized 11 overlapping peptides covering the entire C-protein sequence. The lengths of the peptides were chosen on the basis of the high index of hydrophilic β turns, as judged by secondary-structure prediction analysis according to Chou and Fasman (8) and Hopp and Woods (21) (Fig. 1). These regions are likely to be exposed at the surface and antigenic for B cells. T-cell antigenic sites are postulated to be amphipathic helices with one face predominantly polar and the opposite face predominantly apolar (28). Although antigenicity prediction methods are popular for predicting the locations of virus epitopes, there is evidence that none of the methods achieves a high level of correct prediction. Most workers have used fairly long peptides to search for continuous epitopes in proteins replete with antigenically active regions. In the present studies, long peptides (>25 residues) were synthesized to mimic epitopes on native capsid protein because they have more chance of having a conformation similar to that of the corresponding portion of the native antigen (50). An additional cysteine residue was added to the C-terminal end of peptides C1, C3, C4, C5, C10, and C11 for later conjugation.

T-cell response to C peptides. Sera and PBMC were isolated from seven healthy donors. Six of the seven donors were shown to be seropositive to RV structural proteins in immunoblot analysis using nonreducing conditions (Fig. 2). Four positive sera (MS, AT, RM, and DO) reacted strongly with the C protein. Lymphocyte proliferative responses of human PBMC were determined in vitro by direct stimulation with RV or with increasing doses of the C peptides. PBMC from all six seropositive donors showed significant proliferative response to RV, but weak responses were observed when synthetic peptides were used as stimulating antigens in the fresh PBMC cell proliferative assay (data not shown). One plausible explanation for those findings is that these individuals may have a low frequency of capsid-reactive T

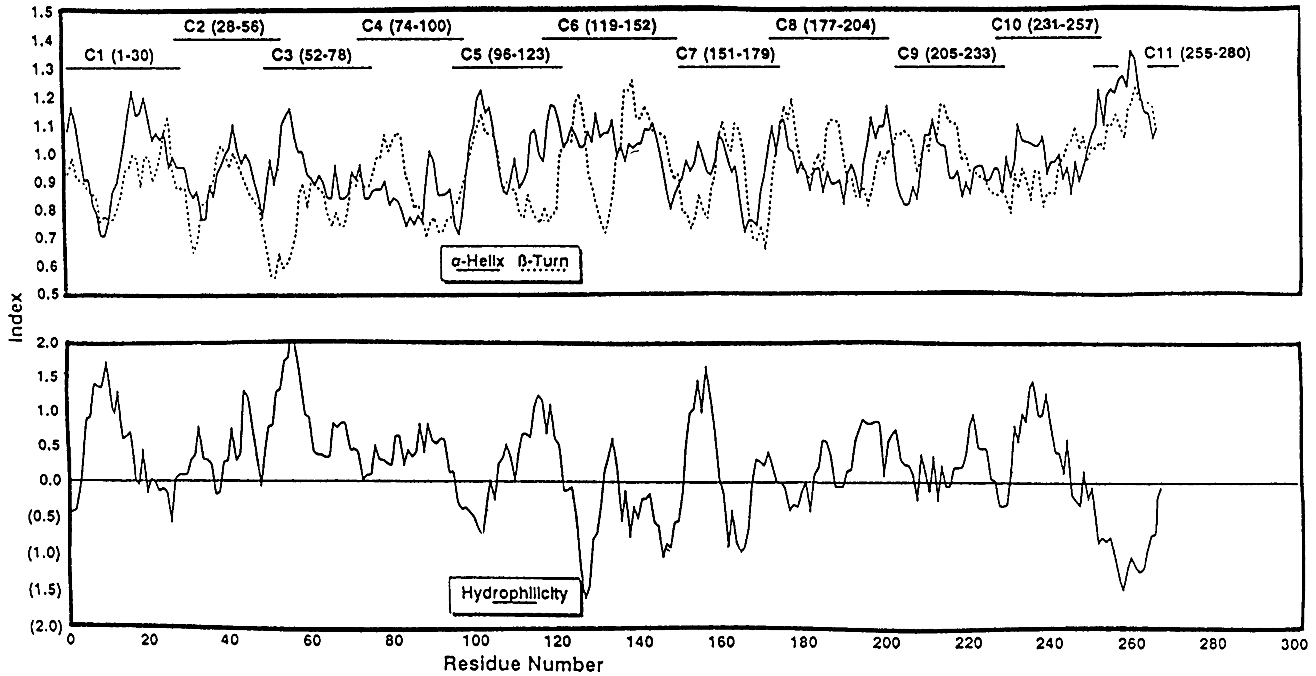

FIG. 1. Predicted structure of RV C protein by conventional structural analysis algorithms. (A) Secondary-structure analysis of local average α-helix and β-turn potentials according to Chou and Fasman (8); (B) hydrophilicity plots predicted by Hopp and Woods (21). The values are derived from the average of heptapeptide windows and are plotted at the midpoint of each segment.

cells in their circulation. To increase the concentration of capsid-specific T cells, fresh PBMC from seropositive individuals were first stimulated with UV-inactivated RV and then cultured in the presence of interleukin-2. After antigen stimulation in vitro, the lymphocyte proliferation response to synthetic peptides was found to be significantly increased in a dose-responsive manner (Fig. 3). Four human T-cell epitopes were mapped to residues 96 to 123 (C5), 119 to 152 (C6), 205 to 233 (C9), and 255 to 280 (C11), since these peptides were shown to be capable of stimulating RV-reactive T-cell lines derived from four seropositive individuals (AT, RM, CM, and DO). Other C peptides were negative in the proliferation assay (Table 1). Of the six lymphocyte cell lines, four (AT, RM, CM, and DO) were found to respond strongly to peptide C11, two (AT and RM) responded to peptides C5 and C6, and one (AT) responded to peptide C9. Two lymphocyte cell lines (SM and TZ) gave no detectable response to any of the 11 capsid peptides. Interestingly, T-cell lines derived from AT and RM recognized multiple T-cell epitopes (four and three C peptides, respectively), while T-cells derived from CM and DO recognized only one peptide, C11. No positive proliferative responses to any synthetic peptides was observed from the seronegative individual (HC). The specificity of these lymphocyte cell lines to RV was further confirmed by using UV-inactivated RV or Epstein-Barr virus as a stimulating antigen in proliferative assays. These T-cell lines showed significant responses only to RV, not to Epstein-Barr virus (data not shown). Immunofluorescent staining of these RV-stimulated lymphocyte cell lines indicated that 95% of the cells were $CD3^+$ and $CD4^+$ and only 3% were $CD8^+$ (data not shown), suggesting that they belong to the helper/inducer T-cell class.

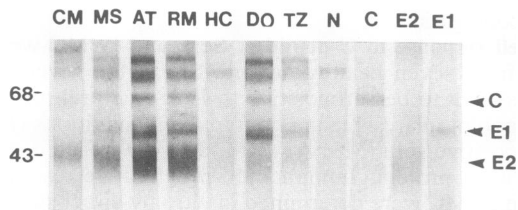

FIG. 2. Immunoblot analysis of participants' sera against M33 RV antigens under nonreducing conditions. Sera (CM, MS, AT, RM, HE, DO, and TZ) were used at a 1:80 dilution. E1, E2, and C refer to the structural proteins of RV detected with monoclonal antibodies against E1, E2, and C proteins, respectively. N is the negative control serum. Relative mobilities of protein standards (in kilodaltons) are indicated at the left.

Reactivity of C peptides with RV-seropositive sera. Numerous studies have demonstrated that good ELISA results can be obtained by coating synthetic peptides directly onto microtiter plates. The peptide ELISA may not work as well for all peptides, since individual synthetic peptides are not covalently bound to the plates. To verify the assay, individual peptides were coated onto the polystyrene microtiter plates under optimal conditions as previously described (17, 47) and probed with peptide-specific rabbit antisera raised against individual C peptides. All peptides were recognized by their respective antipeptide antisera. Reactive titers were >1/1,600 (Table 2). These results establish that all synthetic peptides are adsorbed to the microtiter plates and their antigenic determinants are accessible to antibody interaction. To address the possibility of nonspecific binding between the synthetic peptides and antibodies, synthetic peptides were screened with normal IgGs from rabbits. No nonspecific binding was found.

It was also of interest to determine human B-cell epitopes of RV C protein. To this end, the synthetic peptides were

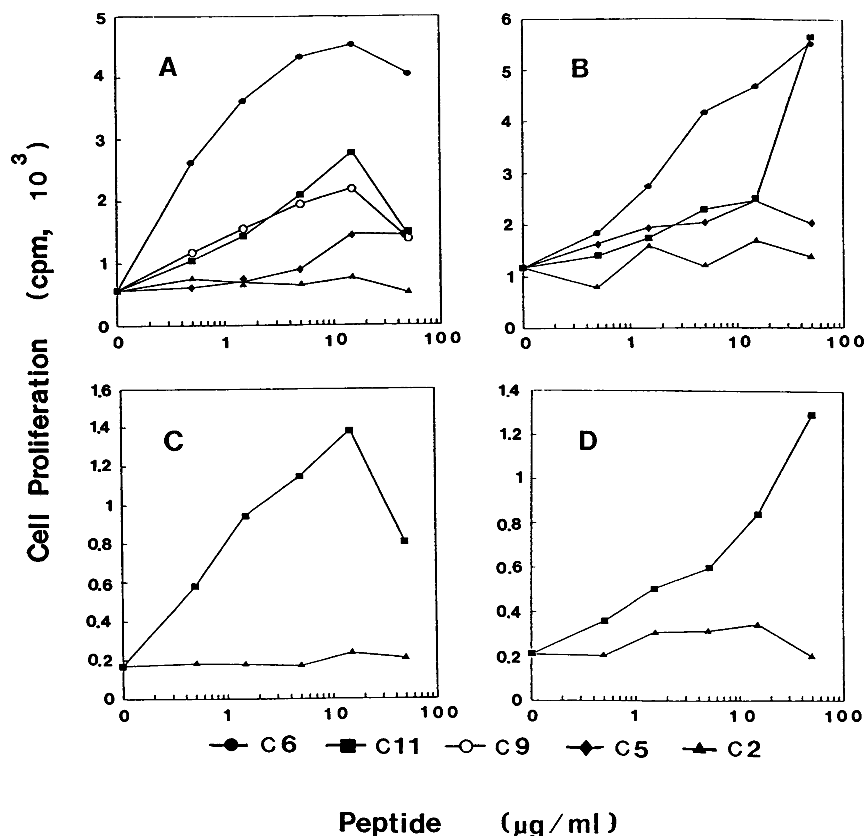

FIG. 3. Recognition of C peptides by RV-reactive T cells from four seropositive individuals. The T-lymphocyte cell lines AT (A), RM (B), CM (C), and DO (D) were tested for their capacity to proliferate at different concentrations of C peptides. The positive responses to peptides C5, C6, C9, and C11 and the negative responses to C2 are shown. Results of the negative responses to other C peptides are not shown.

tested against human RV-positive sera (Table 3). A representative peptide ELISA reactivity is shown in Fig. 4. Sera from all six donors were found to react with peptides C1 and C5. In addition, peptides C4, C8, and C6 reacted with sera from CM and DO, CM and MS, and AT, respectively. It is likely that the immunodominant linear B-cell epitopes of RV C protein are located within residues 1 to 30 (C1) and 96 to 123 (C5). Interestingly, sera of both RM and MS that recognized C protein in immunoblot analysis were found to react weakly with C peptides in the peptide-specific ELISA (Fig. 2 and Table 3). These antibodies might recognize the epitopes that cannot be mimicked by synthetic peptides.

The antigenicity of the synthetic peptides was further assessed by mouse and rabbit antisera raised against C

TABLE 1. Proliferative responses of an RV-reactive T-cell line to synthetic peptides of C protein of RV

Peptide	Amino acids		Cell proliferation indices[a]						
	Positions	Sequence[b]	AT	RM	CM	DO	MS	TZ	HC[c]
C1	1–30	MASTTPITMEDLQKALEAQSRALRAGLAAG(C)	1.2	1.4	1.0	0.9	0.6	1.2	0.9
C2	28–56	(C)AAGASQSRRPRPPRHARAQHLPEMTPAVT	1.0	1.4	1.4	1.0	0.6	1.0	1.2
C3	52–78	TPAVTPEGPAPPRTGAWQRKDWSRAPP(C)	0.9	0.9	1.1	1.0	1.5	1.3	1.0
C4	74–100	SRAPPPPEERQESRSQTPAPKPSRAPP(C)	1.5	1.2	1.1	1.0	1.0	1.1	0.8
C5	96–123	SRAPPQQPQPPRMQTGRGGSAPRPELGP(C)	<u>2.6</u>	<u>2.1</u>	1.1	0.9	1.4	1.1	0.9
C6	119–152	PELGPPTNPFQAAVARGLRPPLHDPDTEAPTEAC	**8.1**	**4.0**	1.3	1.0	1.0	1.3	0.9
C7	151–179	CVTSWLWSEGEGAVFYRVDLHFINLGTP	1.0	1.8	1.0	1.5	0.6	1.5	1.1
C8	177–204	GTPPLDEDGRWDPALMYNPCGPEPPAHV	0.9	1.2	1.4	0.9	0.9	1.4	0.8
C9	205–233	VRAYNQPAGDVRGVWGKGERTYAEQDFRV	<u>3.9</u>	1.5	0.8	0.9	1.1	1.2	0.7
C10	231–257	FRVGGTRWHRLLRMPVRGLDGDTAPLP(C)	1.3	1.2	0.7	0.9	0.6	1.2	0.8
C11	255–280	PLPPHTTERIETRSARHPWRIRFGAP(C)	**5.0**	<u>2.1</u>	**8.2**	**4.0**	0.6	1.1	1.1

[a] RV-induced proliferative responses of a T-cell line from the indicated RV-seropositive individuals, tested for their proliferative responses to C peptides (15 µg/ml). Cell proliferation indices were calculated as described in Materials and Methods. Underlined numbers represent significant proliferative indices between 2 and 4; boldface numbers represent proliferative indices greater than 4.

[b] A, Ala; H, His; P, Pro; W, Trp; C, Cys; I, Ile; Q, Gln; Y, Tyr; D, Asp; K, Lys; R, Arg; E, Glu; L, Leu; S, Ser; F, Phe; M, Met; T, Thr; G, Gly; N, Asn; V, Val;

[c] Seronegative individual used as a control.

TABLE 2. B-cell responses to synthetic peptides in rabbits[a]

Peptide	Density[b]	
	Reducing conditions	Nonreducing conditions
C1	3.21	3.17
C2	0.11	0
C3	0	0.13
C4	0.36	0.76
C5	2.22	1.01
C6	2.08	0.76
C7	0.15	0
C8	0.12	0
C9	0.49	0.55
C10	0.72	0.66
C11	0.23	0.48

[a] In all cases, the ELISA IgG titer was >1,600.
[b] Immunoblot analysis was carried out with rabbit antisera at a 1:100 dilution (Fig. 4). Values represent relative density of the C-protein band in optical density units per millimeter as measured by densitometric tracing. SDS-PAGE was carried out under nonreducing and reducing conditions.

protein or RV. Mouse and rabbit antisera had previously shown to be RV specific, as judged by both ELISA and immunoprecipitation (27). Peptides C1, C3, and C5 reacted strongly with the mouse antisera (Fig. 5A). These peptides represent mouse immunodominant linear B-cell epitopes. Peptides C6 and C9 reacted weakly with the mouse antisera and may be minor B-cell determinants in mouse. Synthetic peptides C5 and C6 were found to be the major B-cell epitopes when the panel of C peptides were screened with rabbit antisera raised against RV particles (Fig. 5B). Peptides C1, C4, C7, C9, and C11 were also recognized by rabbit anti-RV serum.

Immunogenicity of the C peptides in rabbits. The capacity of C peptides to elicit peptide-specific antibody production was examined by immunizing rabbits with individual synthetic peptides emulsified in Freund's complete adjuvant. After three doses (100 μg per injection), IgG antibody responses were tested by using peptide-specific ELISAs. All

TABLE 3. B-cell responses to C peptides in humans

Peptide	ELISA of seropositive individual sera[a]						Density[b]					
	AT	RM	CM	DO	MS	TZ	AT	RM	CM	DO	MS	TZ
C1	+++	+	++	+++	+	++						
C2												
C3												
C4			+	++								
C5	+++	+	++	+++	+	+						
C6	++											
C7												
C8			++		+							
C9												
C10												
C11												
C protein							1.20	0.98	0.76	0.92	0.74	0.52

[a] Responses of sera from six seropositive individuals who displayed positive reactivity indicative of antibody binding to peptide are expressed in terms of relative reactivity as described in Materials and Methods. No positive reactivity was obtained from the serum of seronegative individual HC. IgG antibody reactions were tested at a serum dilution of 1:64.
[b] Data obtained from the immunoblot shown in Fig. 2, representing relative density in optical density units per millimeter of the C-protein band as measured by densitometric tracing.

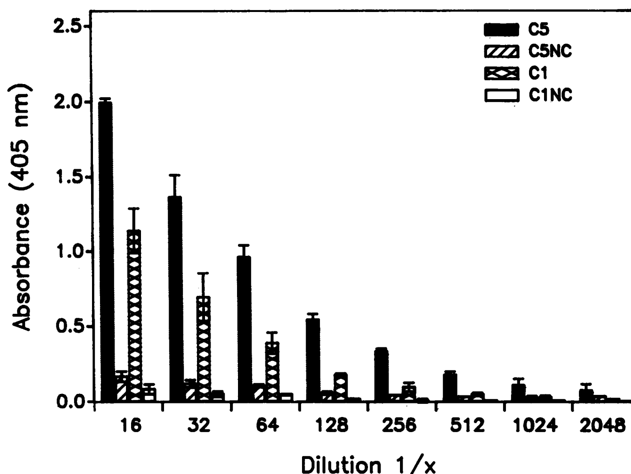

FIG. 4. ELISA using peptides C1 and C5 as antigens in humans. Shown are specific IgG antibody responses of serial twofold dilutions of serum AT to peptides C1 and C5. Cross-hatched and solid columns represent the ELISA absorbance values of the antibody responses to C1 and C5, respectively; hollow and hatched columns represent the ELISA absorbance values of the negative control serum to C1 and C5, respectively.

synthetic peptides were highly immunogenic and induced strong antibody responses (Table 2). Free peptides blocked the binding between peptide and antibodies. Thus, the interaction between peptides and peptide-specific antibodies is highly specific. Cross-reactions of peptide-specific anti-

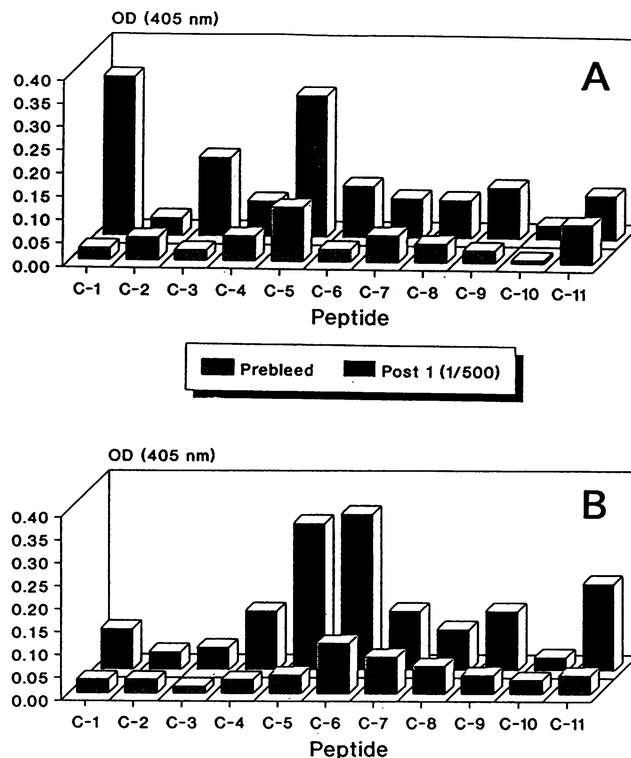

FIG. 5. Peptide ELISA reactivity of mouse anticapsid antisera (A) and of rabbit anti-RV sera (B) with RV C peptides. OD, optical density.

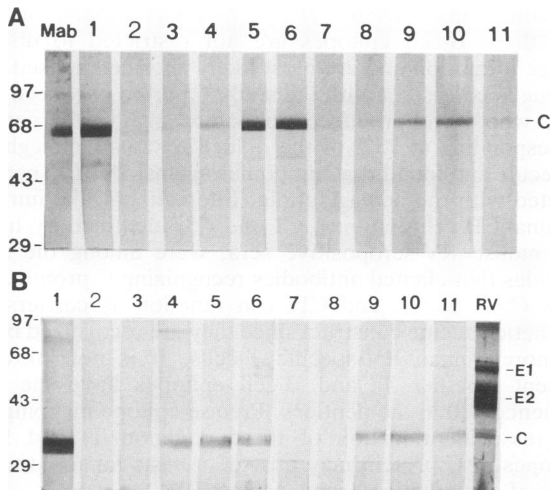

FIG. 6. Immunoblot analysis of the antipeptide sera from rabbits immunized with C peptides. Immunoblot analysis was carried out under nonreducing (A) and reducing (B) conditions. Mab is the blot probed with monoclonal antibodies against C protein. The relative mobilities of protein standards (in kilodaltons) are indicated at the left. E1, E2, and C denote the structural proteins of RV. The antipeptide sera were used at a dilution of 1:100.

sera with C protein were investigated by immunoblot analysis. Purified RV was subjected to SDS-PAGE, and individual viral proteins were electrotransferred to a nitrocellulose membrane. It is worthwhile to point out that under nonreducing conditions (Fig. 6A), C protein migrated as a disulfide-linked dimer of molecular mass 65 kDa (44). Seven of the peptide-specific antisera (anti-C1, -C4, -C5, -C6, -C9, -C10, and -C11) recognized C protein in the western immunoblots under both reducing and nonreducing conditions (Fig. 6). Under reducing conditions, anti-C1 peptide serum exhibited the strongest response (Fig. 6B), whereas anti-C5 and -C6 sera reacted better with the dimeric form of C protein (Fig. 6A). The reactivities of these peptide antisera with C protein under both reducing and nonreducing conditions suggest that the B-cell epitopes are not dependent on conformation and are probably exposed on the protein surface (Fig. 6 and Table 2).

DISCUSSION

Several live, attenuated rubella vaccines have been introduced since 1969 (42). Immunization of infants and susceptible women of child-bearing age against rubella is now a standard public health measure. However, there are still major medical concerns: the reinfection of vaccinees by wild-type RV and the risk of both congenital infection of the fetus and of rubella-associated arthritis arising from vaccination (46). There is a clear requirement for the development of a noninfectious rubella vaccine, for example by genetic manipulation and synthetic methods. Recent studies indicate that immunization of experimental animals with synthetic peptides representing specific regions of viral or bacterial proteins can induce immune responses specific to those proteins and neutralizing their biological functions (34). Thus, synthetic peptides can function as inexpensive and noninfectious candidate vaccines against infectious diseases. Recent developments in fundamental immunology indicate that good and effective immunogens should contain two distinct functional domains, one promoting B-cell interaction for antibody production and a second inducing cognate T-helper cell activity (34–37). It is thus important to define T- and B-cell epitopes for all RV proteins.

Anticapsid antibodies are found in the majority of individuals exposed to RV (11, 44). In this study, human, rabbit, and mouse sera reacted strongly with residues 1 to 30 (C1) and 96 to 123 (C5). Thus, these two regions contain the immunodominant linear B-cell epitopes. No relationship between T- and B-cell responses to peptides is obvious. T-cell lines derived from donors MS and TZ did not respond to C peptides, whereas their sera reacted with peptides C1 and C5. These specific B-cell responses might be induced or helped by the specific T cells whose epitopes were located in other parts of the RV protein than the capsid. Milich et al. (36) found that hepatitis B virus (HBV) core antigen-specific helper T cells could help B cells to produce antibody against both envelope HBV surface and core antigens, even though these antigens were on separate molecules. Recently, an HBV immunogen composed of nucleocapsid T-cell sites and envelope B-cell epitopes has been constructed and shown to be involved in both T- and B-cell responses relevant to the native antigen (35).

T-helper cells participate in the protective immune response by enhancing the production of virus-neutralizing antibodies by B cells, producing lymphokines such as gamma interferon that directly inhibit viral replication and killing infected cells that coexpress viral antigen and major histocompatibility complex (MHC) class II antigens. Although it is known that both T-helper cell proliferation and cytolytic T-lymphocyte responses can be measured during RV infections (3, 24, 29, 51, 53), the exact role of T-helper cells in elimination of RV has not been totally elucidated because of lack of a good RV-infected animal model. Precise locations of epitopes for each viral protein have not been mapped. Overlapping synthetic peptides have permitted successful identification of the antigenic determinants on C protein capable of stimulating RV-specific human T-cell lines. Of the six human RV-specific T-cell lines derived from donors, AT responded to four synthetic peptides (C5, C6, C9, and C11), RM responded to three peptides (C5, C6, and C11), and CM and DO responded only to C11. Cell lines derived from MS and TZ recognized no C peptides. It is possible that T-cell epitopes for these cell lines (MS and TZ) are not contained only within the 11 synthetic peptides. Surface marker analysis by FACS showed that most cells in the lines studied here were $CD4^+$ T-helper cells. The response appeared to be MHC class II restricted, but four donors recognized one immunodominant epitope C11 (residues 255 to 280). This finding is consistent with the results of studies of the T-cell proliferative response to HBV, herpes simplex virus, and influenza virus (2, 12, 37). The role of MHC class II-restricted RV-specific T-cells in RV infection and protection is unknown. A low RV antigen-specific response was shown to be associated with HLA-DW2 (23). The HLA restriction pattern and the relationship of HLA phenotype to T-cell response needs study, as it has been reported that particular HLA phenotypes are immunologically nonresponsive to rubella vaccines and RV infection (23–25). The HLA DR phenotypes of the four individuals (AT, RM, CM, and DO) whose T-cell lines responded to C peptides of RV are 4,7; w9,w53; 1,4,w53; and w9,w53, respectively. We have isolated several T-cell clones specific to peptide C6, C9, C11 from two of them (RM and AT). Preliminary observation of these T-cell clones indicated that

C5 SRAPPQQPQPPRMQTGRGGSAPRPELGP(C)

C6 PELGPPTNPFQAAVARGLRPPLHDPDTEAPTEAC

C9 VRAYNQPAGDVRGVWGKGERTYAEQDFRV

C11 PLPPHTTERIETRSARHPWRIRFGAP(C)

FIG. 7. T-cell motifs in C peptides of RV. The sequences of peptides C5, C6, C9, and C11 are shown. The T-cell motifs are underlined.

recognition of some of these epitopes is associated with HLA-DRw9 or HLA-DR4 (unpublished data).

Recently, two models have been proposed to identify regions of antigens involved in T-cell recognition. Delisi and Berzofsky have found that a large proportion of T lymphocytes appear to react with α-helical amphipathic regions within the amino acid sequence of the antigen (13). On the other hand, the studies of Rothbard and Taylor point to the existence of a four- to five-amino-acid sequence motif in a large number of T-cell antigenic determinants (45). This motif is composed of either a glycine or a charged amino acid residue in the first position, then two or three hydrophobic residues, and either a glycine, a polar, or a charged residue in the last position. Interestingly, none of the identified human T-cell epitopes were predicted as potential T-cell determinants by the amphipathicity algorithm of Delisi and Berzofsky (13). However, these human T-cell epitopes contain one or more T-cell-binding motifs as predicted by Rothbard and Taylor (45) (Fig. 7).

Short synthetic peptides representing B-cell epitopes are generally poor immunogens and need to be coupled to a carrier protein with T-helper cell epitopes in order to induce an antibody response. However, such an approach has limited relevance to vaccine development because of the phenomenon of carrier-induced suppression and the failure to induce priming of a T-cell memory response to the pathogen. Thus, to design a synthetic peptide vaccine, it is necessary to synthesize defined immunogens composing relevant T- and B-cell epitopes. In this study, we were interested in determining whether long peptides containing a high index of hydrophilic β turns, as predicted by secondary structure analysis, could be used as immunogens. Since the minimum length of a T-cell epitope is 8 to 12 amino acids and 6 to 10 residues are required for a B-cell epitope (14, 45), peptides with more than 25 residues were synthesized and tested for immunogenicity. In addition, these peptides were used in the search for functional T-cell epitopes that have not been identified by the human RV-specific T-cell lines because of the limits imposed on T-cell antigen recognition by MHC restriction. All 11 synthetic peptides were highly immunogenic and induced strong antibody responses in rabbits, as judged by the peptide-specific ELISA (Table 2). These results are also consistent with reports by Lerner and collaborators, who suggest that antibodies can be raised against almost any part of a protein by using synthetic peptides as immunogens (26). Cross-reactions of peptide-specific antisera with C protein were demonstrated by immunoblot analysis. Seven of the peptide-specific antisera (anti-C1, -C4, -C5, -C6, -C9, -C10, and -C11) recognized C protein in western blots under both reducing and nonreducing conditions (Fig. 6). The high-affinity binding suggests that these B-cell epitopes are not restricted to disulfide bridge formation and are most likely surface exposed. This finding is consistent with the study reported by Green et al. (19), who have produced antibodies against 20 peptides corresponding to 75% of the influenza virus hemagglutinin molecule and found that antibodies against 18 of 20 peptides reacted with the hemagglutinin. Interestingly, the immunodominant B-cell epitopes, C1 and C5, identified by human and mouse RV-seropositive sera, were among the seven peptides that elicited antibodies recognizing C protein. Peptides C5, C6, C9, and C11 can function as carriers in a synthetic vaccine construct since they are recognized by one or more human RV-specific T-cells. It is not known at present whether T- and B-cell epitopes have the same sequence within the peptides. Precise epitope mapping is in progress. Identification of these common T- and B-cell epitopes of C protein may provide a basis for the development of a synthetic vaccine against RV.

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