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Analysis of Overlapping T- and B-Cell Antigenic Sites on Rubella Virus E1 Envelope Protein

Influence of HLA-DR4 Polymorphism on T-Cell Clonal Recognition

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ABSTRACT: A CTL antigenic site located between residues 273 and 291 of the E1 envelope protein of RV was identified by ^{51}Cr-release assays employing SPs. Two E1-specific CTL clones were examined for immune recognition of RV wild-type and attenuated vaccine strains and recombinant E1 protein. The exact sequence (273–284) recognized by both clones was delineated by using truncated and overlapping SPs covering these residues. The defined T-cell site overlapped almost completely with a virus neutralizing antibody-binding site previously identified with mouse monoclonal and human antibodies. A series of single aa-substituted SP analogues of E1(273–284) was used to define residues critical for T-cell recognition. Using EBV-BL displaying different HLA-DR haplotypes and -DR4 subtypes as targets to determine MHC class II restriction elements, immune recognition by both T-cell clones was shown to be associated with HLA-DR4. Three HLA-DR4 subtypes (DR4Dw13A, DR4Dw13B, and DR4KT2) sharing a common residue, glutamic acid at position 74 in their β1 chains, were able to present SP E1(273–284) to the T-cell clones. *Human Immunology 39, 177–187 (1994)*

ABBREVIATIONS

aa	amino acid
Ag	antigen
CTL	cytotoxic T lymphocyte
EBV-BL	Epstein–Barr virus-transformed B-cell line
FFU	focus-forming units
HCL	HLA homozygous EBV-BL
HLA	human leukocyte antigen
mAb	murine monoclonal antibody
MHC	major histocompatibility complex
PBMNC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
RAA	RV-associated arthritis
rB1BV	recombinant RV E1 protein
rIL2	recombinant interleukin 2
RV	rubella virus
SP	synthetic peptide
TcR	T-cell receptor

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Received (U) July 26, 1993; accepted August 26, 1993.

INTRODUCTION

Rubella virus (RV) infection during early pregnancy may result in spontaneous abortion or severe birth defects and viral persistence in newborns [1]. Live attenuated RV vaccines, which have been used since 1969 to immunize infants and susceptible women of childbearing age, have successfully reduced disease incidence [2]. Nevertheless,

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there are still medical concerns such as primary or secondary vaccine failure and RV vaccine-associated arthritis (RAA). RAA occurs acutely in approximately 10%–15% of previously seronegative women receiving the currently used live attenuated RA27/3 strain of RV vaccine [3]. Of those affected, a significant proportion have been reported to develop chronic and debilitating joint manifestations [1, 3, 4]. Whether this is due to primary immunologic failure leading to RV persistence in joint tissues [5] or virus-induced autoimmunity is still unresolved. Hence, there is an indication for the development of an improved second-generation (peptide, recombinant protein, or modified infectious virus) vaccine by employing only those antigenic regions known to give rise to protective immunity.

In determining the components of a new RV subunit vaccine, it is essential to establish the relevant antigenic sites for RV-specific effector T lymphocytes and protective antibodies [6]. Evidence from other virus studies has indicated a central role for $CD4^+$ T cells in the induction of specific immune responses to virus antigens (Ags) as represented in synthetic peptides (SPs) [7, 8]. It is also known that $CD4^+$ T cells recognize foreign Ags not in their native form but as small peptides that are presented as a complex with major histocompatibility complex (MHC) class II molecules on the surface of Ag-presenting cells [9]. Hence, identification of relevant antigenic sites by using SPs representative of the primary sequence of a candidate vaccine Ag, and characterization of the HLA restriction elements involved in peptide presentation to T cells, could facilitate design of a more effective RV vaccine [10].

RV contains three major antigens: the envelope glycoproteins (E1 and E2) and an internal capsid protein [11, 12]. Four nonoverlapping domains that are binding sites for RV-neutralizing antibodies have been located within residues 213–285 on E1 [12, 14–18]. At least four T-cell antigenic sites within this sequence have also been mapped by using peripheral blood mononuclear cells (PBMNCs) or $CD4^+$ T-cell lines in lymphoproliferation screening assays employing relatively long (16–33 aa) SPs [14, 19–21].

To delineate further the antigenic sites recognized by T and B cells within this E1 region, T-cell lines were derived from RV-reactive human donors by either stimulating freshly isolated PBMNCs with UV-inactivated RV (M33 strain) or by stimulating PBMNCs directly with SPs (19–27 aa long) spanning residues 213–291 of RV E1 protein. From these initial studies, several cytotoxic T-lymphocyte (CTL) clones were isolated by limiting dilution [22, 23], and their specificities were determined in standard ^{51}Cr-release assays, using autologous Epstein–Barr virus-transformed B-cell lines (EBV-BLs) sensitized with SPs as targets. Two T-cell clones were selected for this study: ATRVC2, isolated from a T-cell line stimulated by UV-inactivated RV; and AT177C5, derived from a T-cell line stimulated by the SP E1(273–291). Preliminary investigations revealed that both were specific for an antigenic site (or sites) lying within residues (273–291) of RV E1 protein, a domain previously shown to be the target of RV-neutralizing antibodies [15, 17]. These findings suggested that this region might contain overlapping T- and B-cell antigenic sites that could be potentially useful components of a modified RV vaccine. To understand better the role this antigenic region plays in protective immunity and in the interaction between T and B cells, the exact antigenic sites recognized by each clone and the MHC class II restriction elements involved in their presentation were defined.

MATERIALS AND METHODS

Virus and E1 protein. RV strains M33, RA27/3, and HPV77 were grown in Vero cells and isolated from culture supernatants. Virus stocks were titrated by immunocytochemical focus assays as described previously [23]. Stock solutions containing 5×10^7 focus-forming units (FFU)/ml were inactivated by UV light (254 nm, model UVG-54; UV Products, San Gabriel, CA, USA) at 5 cm for 10 minutes before use. Recombinant RV E1 protein (rE1BV), which was isolated from the culture supernatant of the insect cell line SF9 transformed by recombinant rubella E1 baculovirus, was generously provided by Dr. Shirley Gillam (Department of Pathology, University of British Columbia).

Peptide synthesis. Overlapping SPs (5–24 aa long) encompassing residues 268–291 of E1 protein of RV strain M33 [11] and substituted analogues within the E1 sequence 273–284 were synthesized in an automated peptide synthesizer using established solid-phase methods and purified to 95% homogeneity in reverse-phase high-pressure liquid chromatography by BioChem Immunosystems (Laval, Quebec). Amino acid analyses were performed on each peptide and found to be in good agreement with the theoretical composition.

RV-specific T-cell clones. The T-cell clones (ATRVC2 and AT177C5) used for this study were isolated from two $CD4^+CD8^-$ T-cell lines derived from a single RV-seropositive donor as described previously [22, 24, 25]. Briefly, PBMNCs were incubated with UV-inactivated RV (5×10^5 FFU/ml) or the SP E1(273–291), which was used at a final concentration of 15 μg/ml in complete RPMI-1640 medium 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, which was also supplemented with 10% autologous plasma. After 7 days of incubation at 37°C, the cells were washed three times and resuspended at 10^6 cells/ml in complete RPMI medium supplemented with 10% fetal calf serum (Gibco, Missisauga, Ontario, Canada) and 25 U of human recombinant interleukin 2 (rIL2, Gibco) per milliliter. After 7 days of incubation, RV- or SP-reactive lymphocytes were cloned by limiting dilution in 96-well round-bottom plates (Nunc, Denmark) at an initial density of 0.3 cells per well in the presence of UV-inactivated RV (5×10^5 FFU/ml) or SP (15 μg/ml) with 5% Lymphocult-T-LF (Biotest, Germany), 25 U/ml of rIL2, and γ-irradiated (2500 rad) autologous PBMNCs (5×10^4/well). After 7 days, all of the wells were supplemented with complete RPMI medium containing 5% Lymphocult-T-LF and 50 U/ml rIL2. By days 10–12, clones were visible. Each clone was transferred into fresh medium and distributed to three wells of 96-well flat-bottomed plates. After 5–7 days of incubation, cells originating from each clone were pooled and transferred into a single well of a four-well plate (Nunc) and restimulated with UV-inactivated RV or SP E1(273–291) in the presence of γ-irradiated autologous PBMNCs. At this time, their Ag specificity was determined in ^{51}Cr-release assays, using autologous EBV-transformed B cells sensitized with the SP E1(273–291) as targets as described below. T-cell clones were maintained in culture by stimulating at weekly intervals 2×10^5 T cells with UV-inactivated RV (5×10^5 FFU/ml) or SP E1 (273–291) at a concentration 15 μg/ml in the presence of 1×10^6 autologous, γ-irradiated PBMNCs. Cultures were replenished every 3–5 days with complete medium containing IL2 at the concentrations described above.

EBV-BLs, HLA homozygous EBV-BLs (HCLs) with different HLA-DR phenotypes or DR4 subtypes. Two kinds of EBV-BLs were used as targets in ^{51}Cr-release assays. EBV-BLs AT (autologous to T-cell clones ATRVC2 and AT177C5) and MT, AJ, CM, LM, OZ, DO, and P9B were established in our laboratories from donors with different heterozygous HLA phenotypes (see Tables 3 and 5) as described previously [22, 23]. HCLs, EBV-BLs BSM, YAR, JHa, PF, KB, HOM2, MAT, CRI, Ig-38, MST, SST, and KT2, which are homozygous at the HLA region, with different HLA-DR phenotypes or DR4 subtypes (Tables 4 and 5), were kindly provided by G.T.N. (Virginia Mason Research Center, Seattle, WA, USA).

Cell-mediated cytotoxicity assay. A standard ^{51}Cr-release assay was used to measure CTL responses [22, 23, 25]. For use as CTL targets, EBV-BL or HCL cells (1×10^6) were sensitized by overnight incubation at 37°C with 1×10^6 FFU of UV-inactivated RV, or 5 μg/ml rE1BV, or 10 μM of SP in 1 ml of complete RPMI medium. For assays employing only SPs, incubation time was reduced to 1 hour. After Ag sensitization, cells were washed once with complete medium and labeled with 100 μCi of $NA_2{}^{51}CrO_4$ (Amersham, Oakville, Ontario, Canada) for 1 hour at 37°C. Radiolabeled target cells were washed four times with medium and incubated with T cells at different effector–target ratios for 4 hours in round-bottom 96-well plates (Nunc). All assays were performed in duplicate or triplicate. Percent specific cytotoxicity was calculated by the formula 100 [(ER − SR)/(MR − SR)], where ER (experimental ^{51}Cr release) = mean counts per minute (cpm) released into the supernatant in the presence of T cells using 5×10^3 target cells; SR (spontaneous ^{51}Cr release) = mean cpm in the absence of T cells determined in four replicate samples; and MR (maximal ^{51}Cr release) = mean cpm in supernatant of target cells incubated with 0.5% Nonidet-P40 detergent (Sigma, St. Louis, MO, USA) determined in four replicates. SR was always <25% of MR.

mAbs. Anti-DR monoclonal antibody (mAb) P4.1 [26] and anti-DQ mAb 200.1 [27] were also provided by G.T.N. Ascites fluids containing these mAbs were used to inhibit CTL responses in ^{51}Cr-release assays by incubating SP E1(273–284)-sensitized target cells at final dilutions of 1:200 for 30 minutes before adding CTL cells.

Polymerase chain reaction (PCR) and sequence analysis. One microgram of genomic DNA from EBV-BL AT was amplified by PCR, using DR4 group-specific primers BN17 (5′-CCGGAACCTCTTGGAGCAGGTTAAACA-3′) and BN22 (5′-CGTTCGAATGCACTGTGAAGCTCTC-3′) with 1-minute cycle times and a 94°, 65°, and 72°C temperature cycle. The PCR product was directly sequenced by the dideoxy-chain termination method using Taq polymerase and the BN22 oligonucleotide as the primer.

RESULTS

Immune recognition of E1 SPs, rE1BV, and RV strains M33, RA27/3, and HPV77 by cytotoxic T-cell clones. T-cell clones ATRVC2 and AT177C5 were determined to be $CD4^+CD8^-$ T cells by flow-cytometric analysis (data not shown). Both clones were tested in cytotoxicity assays for recognition of recombinant E1 protein, wild-type RV (M33) and attenuated RV vaccine strains (RA27/3 and HPV77). Both clones exhibited strong cytotoxicity for autologous EBV-BL targets sensitized with the SP E1(273–291) and, to a lesser extent, also killed the same targets sensitized with rE1BV or the three

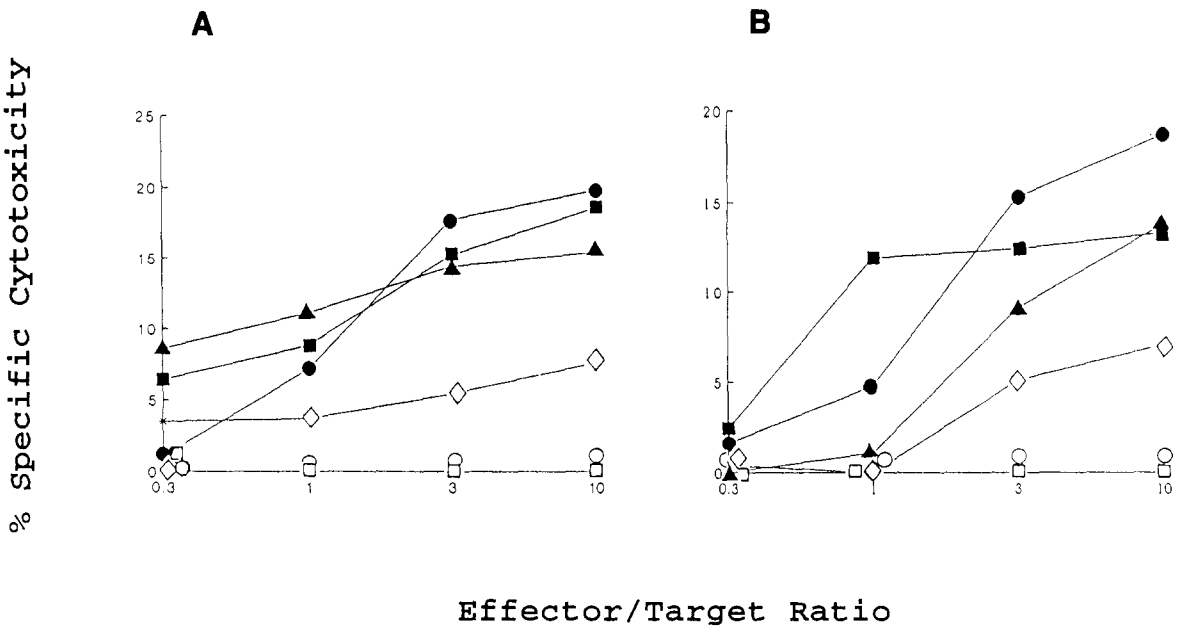

FIGURE 1 Recognition of RV wild strain M33, attenuated strains RA27/3 and HPV77, and rE1BV. CTL clones ATRVC2 (A) and AT177C5 (B) were tested in standard ^{51}Cr-release assays. Target cells used were autologous EBV-BL AT cells incubated with UV-inactivated RV M33 (●), HPV77 (■), and RA27/3 (◇) at 5×10^5 FFU/ml or rE1BV at 5 μg/ml (▲). AT cells alone (□) or cells incubated with culture supernatant of cell line SF9 transformed by wild-type baculovirus (○) were used as controls.

strains of UV-inactivated whole RV (Fig. 1 and Table 1). In the latter case, the weakest cytotoxicity was observed with the RA27/3 vaccine strain (Fig. 1).

Fine mapping of the T-cell antigenic site recognized by RV E1-specific CTL clones. To identify the exact antigenic site recognized by CTL clones ATRVC2 and AT177C5,

TABLE 1 Relative capacities of synthetic peptides within E1 residues, 268–291, to elicit specific cytotoxicity of T-cell clones ATRVC2 and AT177C5[a]

		% Specific cytotoxicity[b]							
		ATRVC2				AT16C5			
RV E1 SP positions	Amino acid sequence	10	3	1	0.3	10	3	1	0.3
		(Effector–target ratio)							
E1(268–291)	APGPGEVWVTPVIGSARKCGLHI	70.7	58.1	40.2	21.3	71.2	66.8	35.9	16.4
E1(273–291)	EVWVTPVIGSQARKCGLHI	77.0	56.7	42.4	16.1	73.9	60.6	43.4	14.7
E1(272–285)	GEVWVTPVIGSQAR	79.7	64.0	44.5	18.5	70.4	61.9	41.0	13.0
E1(273–284)	EVWVTPVIGSQA	77.7	68.3	40.2	25.2	71.6	65.9	43.7	23.0
E1(272–282)	GEVWVTPVIGS	51.3	18.0	1.9	0	54.5	14.0	0.4	0
E1(272–281)	GEVWVTPVIG	3.9	0	0	0	5.3	2.7	2.5	1.9
E1(273–282)	EVWVTPVIGS	4.7	2.7	2.5	1.9	4.2	2.0	1.1	0.4
E1(273–281)	EVWVTPVIG	3.7	0	0	0	0	0	0	0
E1(273–277)	EVWVT	0	0	0	0	0	0	0	0
E1(270–279)	GPGEVWVTPV	9.3	6.7	2.5	1.1	3.7	6.1	4.5	4.7
E1(276–285)	VTPVIGSQAR	0	0	0	0	0	0	0	0
E1(277–288)	TPVIGSQARKCG	0	0.8	1.2	0	1.5	0	0	0
E1(280–291)	IGSQARKCGLHI	0	1.9	0	0	0.7	1.7	3.3	1.3
None		0	0	0	0	0	0	0	0

[a] For use as cytotoxic targets, autologous EBV-BL AT cells were incubated with individual peptides at 10 μM for 1 hour before adding T cells.

[b] Cytotoxicity was determined at different effector–target ratios in ^{51}Cr-release assays.

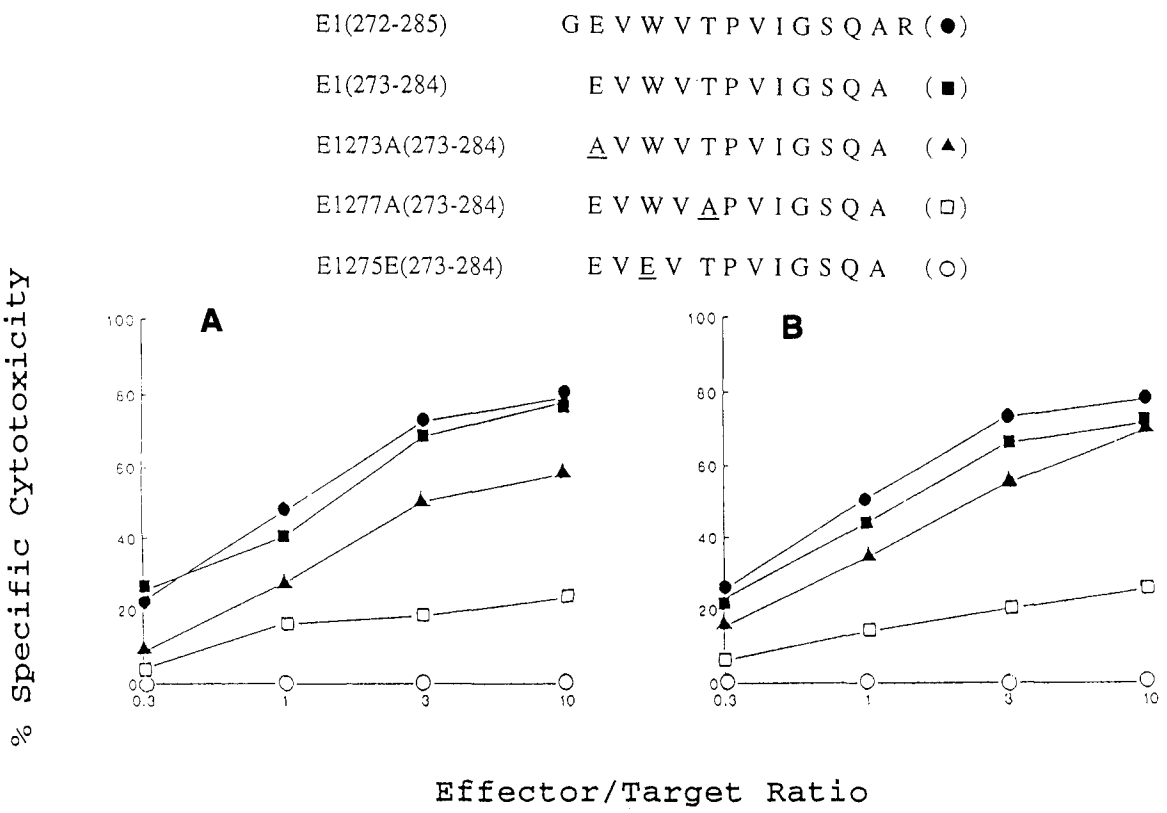

FIGURE 2 The effects of single amino acid substitutions of SP E1(273–284) on cytotoxic responses of T-cell clones ATRVC2 and AT177C5. CTL clones ATRVC2 (**A**) and AT177C5 (**B**) were tested in ^{51}Cr-release assay for specific cytotoxicity directed to SP E1(272–285) (●), E1(273–284) (■), and the single amino-acid-substituted SP analogues E1273A(273–284) (▲), E1277A(273–284) (□), and E1275E(273–284) (○). Autologous EBV-BL (AT) cells sensitized with these SPs were used as targets. The sequences of SP E1(273–284) and its substituted analogues are shown at the top. Underlined characters represent the amino acid substitutions at the indicated positions.

a nested set of truncated SPs within the sequence E1(268–291) was prepared and each peptide was tested in ^{51}Cr-release assays as described above. Because the SPs varied in length, all were adjusted to a concentration of 10 μM for sensitizing target cells. SPs E1(268–291), E1(272–285) and E1(273–284) were found to be equally efficient in eliciting cytotoxicity, as compared with the originally identified antigenic 19-mer, SP E1(273–291) (Table 1). However, a shorter SP, E1(272–282), elicited lower levels of killing by T-cell clones ATRVC2 and AT177C5. Sensitization of target cells with shorter peptides, such as E1(270–279), E1(272–281), E1(273–281), E1(273–282), E1(273–277), E1(276–285), E1(277–288), and E1(280–291), did not result in lysis (Table 1). Single aa-substituted SP analogues of E1(273–284) including E1273A(273–284), in which alanine (A) was substituted for glutamic acid (E) at position 273; E1277A(273–284), in which A was substituted for threonine (T) at position 277; and E1282T(273–284), in which T was substituted for serine (S) at position 282, were also found to elicit lower cytotoxicity than the natural sequence (Fig. 2 and Table 2). Charge substitutions, however, such as glutamic (E) or aspartic (D) acids for tryptophan (W) at position 275, or for valine (V) at position 279 or for isoleucine (I) at position 280 or for glutamine (Q) at position 283, or substitution of nonpolar or relatively larger residues such as tyrosine (Y) or tryptophan (W) for T at position 277, or for S at position 282, completely abolished the specific cytotoxicity (Fig. 2 and Table 2).

Determination of HLA class II restriction of immune recognition of E1(273–284) by CTL clones ATRVC2 and AT177C5. As both ATRVC2 and AT177C5 were shown to be CD4$^+$ CTLs, HLA class II restriction elements associated with the immune recognition of the antigenic site defined above were determined. mAbs (P4.1 and 200.1) specific for determinants on HLA-DR and -DQ, respectively, were tested in ^{51}Cr-release assays for their abilities to block the presentation of SP E1(273–284) by the autologous EBV-BL, AT. SP-specific killing mediated by clones ATRVC2 and AT177C5 was inhibited by preincubating SP-sensitized targets with anti-HLA-DR mAb P4.1 but not by anti-HLA-DQ mAb 200.1 (Fig. 3), suggesting that an HLA-DR restriction

TABLE 2 Cytotoxicity of T-cell clones for autologous EBV-BL sensitized with single amino-acid-substituted SP analogues of E1(273–284)

		% Specific cytotoxicity[a]							
		AT177C5				ATRVC2			
		10	3	1	0.3	10	3	1	0.3
SP analogue[b]	Sequence	(Effector–target ratio)							
E1(273–284)	EVWVTPVIGSQA	76.9	53.6	38.3	10.4	93.8	58.5	41.5	4.6
E1283D	EVWVTPVIGS**D**A	7.7	0	0.5	0	3.9	0	0	0
E1282T	EVWVTPVIG**T**QA	55.9	44.7	20.7	15.6	68.0	33.9	21.6	7.3
E1282W	EVWVTPVEG**W**QA	0	0	0	0	0	0	0	0
E1280E	EVWVTPVEGSQA	5.2	0.3	0	5.7	8.7	0	6.4	3.0
E1279E	EVWVTP**E**IGSQA	0	0	0	5.3	0.9	0	3.3	9.0
E1277Y	EVWV**Y**PVIGSQA	0	0	0	0	0	0	2.6	0

[a] Cytotoxicity was determined at different effector–target ratios in ^{51}Cr-release assays.

[b] For use as targets in cytotoxicity assays, autologous EBV-BL AT cells were presensitized with peptide analogues at a concentration of 10 μM. Bold represents amino acid substitutions.

element was involved in presentation of SP E1(273–284). More detailed analysis of the restriction elements involved was performed by using EBV-BLs expressing different HLA-DR phenotypes (Tables 3 and 4) as targets in cytotoxicity assays. Results showed that the antigenic site defined for the T-cell clones was recognized only in the context of HLA-DR4, which was the phenotype of the autologous EBV-BL, AT, and was also shared by the heterologous line, MT (Table 3), and homologous line, JHa (Table 4). Other heterologous EBV-BLs expressing HLA-DR4 (lines AJ and CM, whose data are shown in Table 3) were unable to present E1(273–284) to the T-cell clones suggesting that, in this case, immune recognition was likely to be DR4 subtype specific. By using HCLs and EBV-BL with different HLA-DR4 subtypes sensitized with E1(273–284) as targets in cytotoxicity assays, it was determined that only three HCls—SST(DR4Dw13A), JHa(DR4Dw13B), and KT2(DR4KT2)—could present E1(273–284) to clones ATRVC2 and AT177C5 (Table 5).

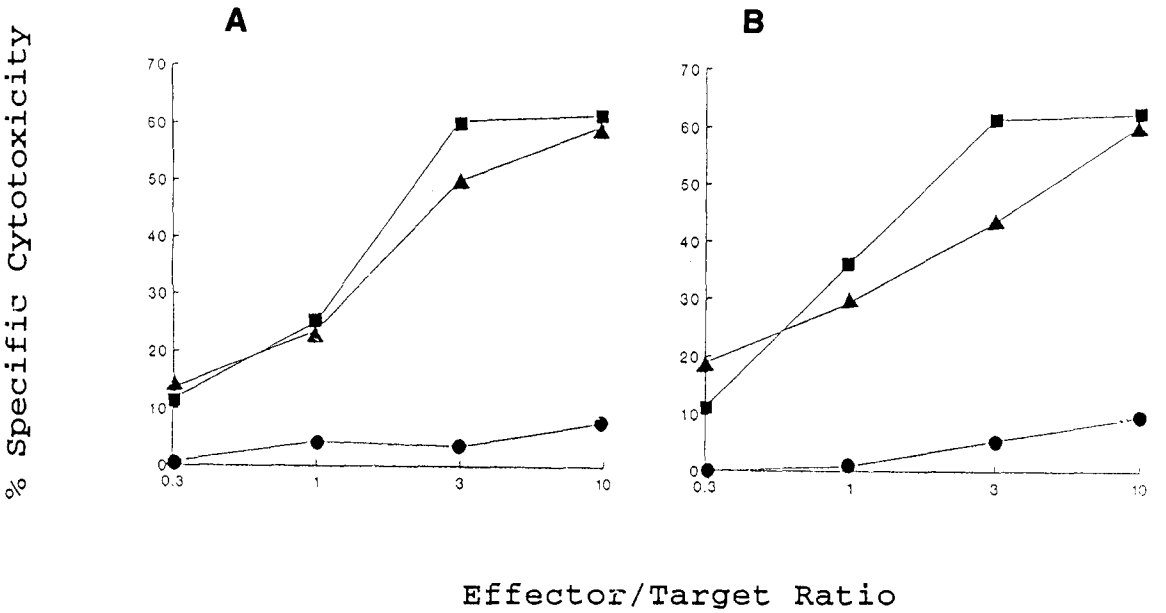

FIGURE 3 mAb inhibition of CTL responses directed to peptide E1(273–284). mAb P4 (anti-HLA-DR) (●) and mAb (anti-HLA-DQ) (■) were incubated with SP E1(273–284)-sensitized target cells at 1:200 dilution at 37°C for 30 minutes before adding of CTLs ATRVC2 (**A**) or AT177C5 (**B**). Medium alone (▲) was used as control.

Sequence analysis of HLA-DR β1 chain of autologous EBV-BL AT. As the HLA-DR4 subtypes DR4B1*0403, DRB1*0407, and DR4B1*0406 share a common resi-

TABLE 3 Cytotoxicity of CTL clones ATRVC2 and AT177C5 for the RV SP E1(273–284) presented by EBV-BL targets with different HLA phenotypes

Targets[a] EBV-BL	HLA DR	HLA DQ	% Specific cytotoxicity ATRVC2 10	ATRVC2 3	ATRVC2 1	ATRVC2 0.3	AT177C5 10	AT177C5 3	AT177C5 1	AT177C5 0.3
			(Effector–target ratio)							
AT	4,7,w53	w3	69.6	51.5	33.9	14.7	63.5	63.7	32.7	10.6
MT	4,w53	w3	66.9	47.2	25.4	10.5	71.1	44.4	26.9	12.2
AJ	4,w17,w52,w53	w2,w7	6.4	3.0	0.2	0	6.3	5.8	3.0	0
CM	1,4,w53	w1,w3	0	0	0	0.3	0.8	1.0	0	0.2
LM	1,w11,w53	w1,w7	0	0.8	0	0	0.5	0.6	0	0
OZ	1,w13,w52	w1	4.3	0.7	0.1	0.4	4.0	0.6	2.6	0.6
DO	w9,w53	w3	2.9	0.5	2.6	0	0	0	0.7	2.3

[a] Target cells were incubated with SP E1(273–284) at a concentration 10 μM for 1 hour before adding T cells.

due, glutamic acid (E) at position 74 in their β1 chains, the DR β1 sequence of the autologous EBV-BL, AT, was determined to see whether it also contained (E) at position 74. This was determined to be the case and the DNA sequence from residues 1 to 86 of the first domain on HLA-DR β1 chain of AT was shown to be identical to that of DR4B1*0403 [28].

DISCUSSION

Two RV-specific HLA-DR-restricted, $CD4^+CD8^-$ CTL clones, ATRVC2 and AT177C5, were generated from the PBMNCs of a single HLA-DR4/DR7 subject by in vitro stimulation with either UV-inactivated M33 strain RV (ATRVC2) or the SP E1(273–291) (AT177C5). Both showed identical antigenic specificity for E1(273–284) and behaved as cytotoxic T cells. If this phenotype is predominant in the in vivo response to this antigenic region, then these cells could function in either immune protection (by direct elimination of RV-infected cells) or adverse immunoreactivity (by destruction of synovial cells persistently infected with RV). These clones exhibited identical specificity despite having been generated with different antigenic stimuli. Moreover, both recognized not only the E1 SPs but also naturally processed peptides from wild-type (M33) and attenuated RV vaccine strains (RA27/3 and HPV77), as well as recombinant RV E1 protein. Although all three RV strains are considered to be completely homologous within residues 273–291 [29, 30], weaker cytotoxicity was observed

TABLE 4 Determination of HLA-DR restriction elements involved in recognition of E1(273–284) by CTL clones ATRVC2 and AT177C5

Targets HCL	HLA-DR phenotype	% Specific cytotoxicity[a] ATRVC2 10	ATRVC2 3	ATRVC2 1	ATRVC2 0.3	AT177C5 10	AT177C5 3	AT177C5 1	AT177C5 0.3
		(Effector–target ratio)							
HOM2	DR1	0	0	0	0	0	0	0	0
MST	DR2	0	0	0	0	0	0	0	0
MAT	DR3	0	1.0	1.7	2.9	0	0	0	0
JHa	DR4	67.0	51.6	22.4	15.2	71.1	55.4	30.3	18.2
Ig38	DR5	0	0	0	0	0	0	0	0
CRI	DR6	1.8	5.5	2.7	0.1	1.8	0.7	0	0
IBw9	DR7	0	0	0	0	0	0	0	0
AT[b]	DR4,DR7	69.6	51.5	33.9	14.7	63.5	60.7	32.7	10.6

[a] Target cells, HCLs, expressing different HLA-DR phenotypes were sensitized with SP E1(273–284) at a concentration of 10 μM for 1 hour before adding T cells.

[b] Autologous EBV-BL of the T-cell clones ATRVC2 and AT177C5.

TABLE 5 HLA-DR4 subtype restriction of cytotoxic responses of T-cell clones ATRVC2 and AT177C5 to SP E1(273–284)[a]

		HLA-DR4 β-chain residue[c]						% Specific cytotoxicity[b] ATRVC2				AT177C5			
		57	67	70	71	74	86	10	3	1	0.3	10	3	1	0.3
Target	Allele	(Position)						(Effector–target ratio)							
BSM	DR4Dw4 (DRB1*0401)	D	L	Q	K	A	G	0	0	0	0	0	0	0	0.2
YAR	DR4Dw10 (DRB1*0402)	—	I	D	E	—	V	0	0	2.4	2.4	0	1.5	3.4	0.4
SST	DR4Dw13A (DRB1*0403)	—	—	—	R	**E**	V	76.2	60.2	36.8	18.9	72.2	53.8	39.9	19.2
JHa	DR4Dw13B (DRB1*0407)	—	—	—	R	**E**	—	59.0	34.0	21.6	5.9	65.7	66.1	38.2	21.8
PF	DR4Dw14A (DRB1*0404)	—	—	—	R	—	V	0	0	1.3	1.6	0	0.4	4.0	1.8
KB	DR4Dw15 (DRB1*0405)	S	—	—	R	—	—	0	0	0.5	0	0	2.0	0	0
KT2	DR4KT2 (DRB1*0406)	—	—	—	R	**E**	V	55.3	51.6	43.3	17.1	66.3	60.9	37.3	14.3
P9B[b]	DR4Dw14B (DRB1*0408)	—	—	—	R	—	—	4.3	1.7	3.6	2.2	7.4	3.8	0	0.7
AT	DR4 (DRB1*0403)	—	—	—	R	**E**	V	78.8	53.9	30.5	20.9	75.0	64.5	46.5	20.1

[a] Target cells with different HLA-DR4 subtypes were incubated with SP E1 (273–283) at a concentration of 10 μM for 1 hour before adding T cells.

[b] EBV-BL P9B is heterozyous at HLA-DR (DR4/DR2) region.

[c] The sequences are shown using the single-letter code, with dashes representing identity to the DR4Dw4 sequence. Residues shown in bold indicate the unique differences between DR4Dw13A, DR4Dw13B, DR4TK2, and the other DR4 subtypes.

when target cells were sensitized with RA27/3. Flanking amino acids may play a critical role in recognition of a given peptide sequence, presumably by influencing Ag processing, peptide conformation, and affinity for MHC binding [31]. Sequence analysis for RV structural proteins has revealed 12 aa substitutions in RA27/3 E1 protein relative to M33 strain [29], while only three substitutions were reported for HPV77 E1 [30]. Hence, the greater sequence variability of RA27/3 E1 may have influenced Ag processing.

To delineate which residues within E1(273–291) were critical for immune recognition by these CTL clones, truncated SPs spanning this region were compared for their capacity to target specific cytotoxicity. Results showed that E1(273–284) was the shortest sequence capable of eliciting maximal cytotoxicity, while E1(272–282), the smallest antigenic SP evaluated, induced somewhat lower levels of killing. Rothbard and Taylor [32] and Rothbard and Gefter [33] have identified a primary sequence motif characteristic of T-cell antigenic sites consisting of 4–5 aa, including a charged residue or glycine, followed by 2–3 hydrophobic aa, then either a polar residue or glycine. Within E1(273–284), such a motif (EVWVT) was identified (Fig. 4). To assess the role played by individual residues, four single aa-substituted analogues of SP E1(273–284) were evaluated in cytotoxicity assays. Results showed that substituting a hydrophobic residue for a charged or polar one at position 273 or 277 substantially reduced cytotoxicity, indicating that these amino acids are important for recognition. Moreover, replacement of a nonpolar aa (W) at position 275 with an acidic aa (E) or replacement of a polar aa (T) with relatively larger-size residue (Y) at position 277 obliterated specific cytotoxicity. Hence, W-275 and T-277 appear to play a critical role in immune recognition of this sequence by these particular CTL clones. De Lisi and Berzofsky have proposed another model in which T-cell determinants correlated with amphipathic α-helic structures in antigenic proteins [34]. The RV E1 sequence, EVWVT, is also located at the maximum of α-helix indexes within residues 273–291 as determined from α-helix and β-turn potentials and hydrophilicity plots of E1 [14].

mAb experiments and cytotoxicity assays performed with different EBV-BLs confirmed that the HLA-DR4 subtypes DR4Dw13A (DRB1*0403), DR4Dw13B (DRB1*0407), and DR4KT2 (DRB1*0406) could serve as restriction elements for presentation of E1(273–284) in this case. Furthermore, the DR β1 chain of the autologous EBV-BL, AT, was shown to have a nucleic acid

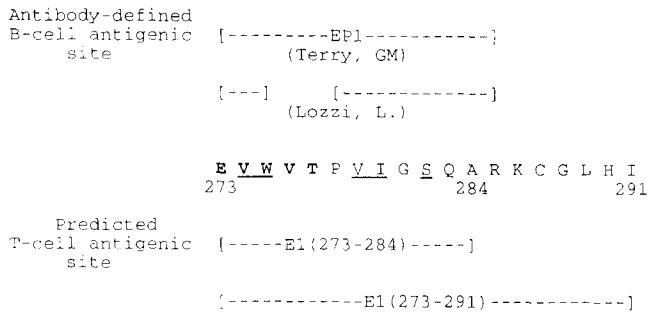

FIGURE 4 Proposed overlapping T- and B-cell antigenic sites on RV E1 protein. A schematic summary of the mouse mAb- and human Ab-defined neutralizing antibody-binding domain (15, 17) and an overlapping T-cell antigenic site E1(273–284), as identified in this study is shown. **Bold letters** indicate the amino acids predicted to be involved with T-cell recognition. Underlined amino acids indicate suggested HLA-DR-binding residues.

sequence identical to DRB1*0403. The molecular diversity of HLA-DR4 haplotypes is well known [35], and at least 12 DR4 subtypes expressing different class II β1 chains have been identified [28]. It is believed that the critical residues involved in Ag presentation by DR4 subtypes are located on the first domain of the HLA-DR4 β1 chain, which shows the most variability [28, 35]. DR4Dw13A, DR4Dw13B, and DR4KT2, which were able to present E1(273–284) to the T-cell clones, differed significantly from the other DR4 subtypes at position 74, with a unique substitution (E for A). Such a charge change could influence immune recognition either by modifying peptide conformation or its interaction with the T-cell receptor (TcR). Alanine (A)-74 in the DR4 β1 chain has been reported to be important in T-cell allo- or autoreactivity [36, 37]. A proposed HLA-DR-binding model consists of a core sequence of six amino acids involving a large hydrophobic residue separated by four amino acids from a small residue [38, 39]. O'Sullivan et al. [40, 41] also proposed a similar nine-residue motif composed of an aromatic or hydrophobic aa in position 1, followed by a noncharged and relatively small residue in position 6, and another relatively hydrophobic one in position 9. As predicted from these models and from the data presented herein, it is highly likely that the V-274 or W-275 and the V-279 or I-280, respectively, represent the first and second hydrophobic anchor residues for binding to HLA-DR4. The third or more allele-specific anchor residue is likely the S-282 (Fig. 4). Hence, these appear to be critical in both binding of the epitope, E1(273–284) to HLA-DR4 and its recognition by the T-cell clones in this study (Fig. 2 and Table 2). Investigations are in progress to confirm the interaction of these critical residues with the HLA-DR-binding groove, or TcR. The results will determine the influence of residue 74-E in DR4 β1 chain on peptide binding.

The results of this clonal analysis revealed an antigenic site E1(273–284), that appeared to be highly restricted in its presentation to T cells and, as such, may not be very good for incorporation into a new RV vaccine. Yet residues 273–284 lie within a broader region of RV E1(268–291), which appears to be widely recognized on a population basis by both T cells [19, 21] and antibodies [15, 17] from individuals of diverse MHC backgrounds. Why would such an antigenic site located within a predicted immunodominant region exhibit such high specificity of MHC restriction? One possible explanation is that the HLA-DR4 subtype-restricted sequence identified here represents only one of several clustered antigenic sites lying within E1 residues 268–291. Alternatively, there may exist in the human T-cell repertoire other TcRs capable of recognizing residues 273–284 in the context of other MHC class II molecules. Investigations are currently under way to determine whether this is the case. It is notable, however, that the HLA-DR4 phenotype has been associated with autoimmunity in other disease systems [42], as has restricted TcR V (β) gene usage [43]. It is also of interest that the antigenic site recognized by the CTL clones described here overlaps almost completely with a previously defined [17] neutralizing antibody-binding domain (EP1), suggesting that the apparent overlap between a class II HLA-restricted T-cell antigenic site and an antibody recognition site may not simply be fortuitous but may reflect a mechanistic link between Ag presentation and T-cell help for antibody synthesis. Overlapping B- and T-cell determinants could facilitate Ag uptake by B cells bearing surface Ag receptors of the appropriate specificity, followed by Ag processing and presentation by the B-cell HLA class II to the T cell, forming an "antigen bridge" to focus the appropriate T cells onto B cells [44].

In conclusion, delineation of a T-cell antigenic site within the RV E1 sequence (273–284), which overlaps almost completely with the previously identified EP1-neutralizing antibody-binding domain on this RV envelope protein, suggests that this region could have functional significance in immune protection mediated by both T cells and antibodies. If the outcome of the in vivo immune response to this epitope is immune protection, then this region would be a useful candidate for developing an effective subunit or recombinant infectious virion vaccine. The results of this study suggest, however, that the influence of MHC and TcR polymorphism on T-cell recognition of SP sequences should be considered in vaccine design. Future studies will determine whether this sequence may be recognized by other human TcRs in the context of other MHC class II molecules, and the phenotypes of the responding T cells, to assess its suit-

ability as a component of second-generation RV vaccines. These investigations will also assess its in vivo immunogenicity for inducing protective or nonprotective T- and B-cell responses.

ACKNOWLEDGMENTS

This work was supported by research grant MT-7351 (Medical Research Council of Canada) and a research grant from the Mary Pack Research Fund (The Arthritis Society, B.C. and Yukon Division). A.J.T. is a recipient of the Career Investigatorship award of the B.C. Children's Hospital Foundation. We gratefully acknowledge Dr. Shirley Gillam, Department of Pathology, University of British Columbia, Canada, for providing recombinant rubella E1 protein.

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