Characterization of the Specificity and Genetic Restriction of Human CD4+ Cytotoxic T Cell Clones Reactive to Capsid Antigen of Rubella Virus

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Using 11 overlapping synthetic peptides covering more than 95% of the amino acid sequence of capsid protein of rubella virus, 7 CD4+ T cell clones (R10, R11, R18, A2, A10, A11, and A12) isolated from 2 rubella seropositive donors reacted strongly to rubella capsid peptides C6 (residues 119–152), C9 (residues 205–233), or C11 (residues 255–280), respectively, in both proliferation and cytotoxicity assay. Truncated peptides C6E (residues 125–139), C9B (residues 205–216), and C11E (residues 260–272) were shown to be involved directly to the T cell determinants of C6, C9, and C11, respectively. Genetic restriction of these T cell clones was analyzed by using human cell lines with various HLA-DR phenotypes as targets and/or antigen-presenting cells in cytotoxicity assay and/or proliferation assays. The results indicated that the recognition of peptide C6 by T cell clones (R11 and R18) was associated with DRw9 molecule, while the HLA restriction element of the responses of other T cell clones (A2 and A11, A10, and A12) that reacted with peptide C9 or C11 was DR4 molecule. However, there may be a cross-recognition by the T cell clone (A12) between DR1 and DR4 subtypes. © 1992 Academic Press, Inc.

Rubella virus (RV) is a common human pathogen. RV infections during pregnancy may result in spontaneous abortion and congenital defects or viral persistence in newborns (Sandra et al., 1985). Several live, attenuated rubella vaccines have been introduced since 1969 (Perfins, 1985). Immunization of infants and susceptible women of child-bearing age against RV 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 rubella-associated arthritis arising from vaccination (Chantler et al., 1982; Fraser et al., 1983; Sandra et al., 1985). There is a clear requirement for the development of a noninfectious rubella vaccine. Recent studies indicate that synthetic peptides can function as inexpensive and noninfectious candidate vaccines against infectious diseases (Milich, 1988).

It is important to know the major human T cell sites when selecting short synthetic peptides as components of potential subunit vaccines. Evidence from many virus studies has indicated a central role for CD4+ rather than CD8+ T cells in the induction of specific immune responses to virus antigens such as subunit peptides (Good et al., 1988; Korner et al., 1991; Fayolle et al., 1991). It is known that CD4+ T cells rec-

ognize foreign antigens (Ag) not in their native form but as small peptides that are produced from processed Ag within Ag-presenting cells (APCs) and presented at the cell surface as a complex with major histocompatibility complex (MHC) class II molecules (Milich et al., 1987; Berzofsky, 1989). Thus, identifying these small peptide epitopes within the primary sequence of a candidate vaccine Ag and defining their genetic restriction of recognition by T lymphocytes could facilitate effective vaccine design (Milich et al., 1987; Wallace et al., 1991).

RV is an enveloped RNA virus with two envelope glycoproteins (E1 and E2) on the virion surface and a capsid protein in the RNA core (Oker-Blom et al., 1983). We have synthesized 49 synthetic peptides comprising approximately 95% of the amino acid sequences of the structural proteins E1 (23 peptides), E2 (15 peptides), and C (11 peptides) of RV strain M33 (Clarke et al., 1987) and initiated a series of experiments to search for peptide-specific T cell responses in individuals naturally infected with RV. We have showed that C peptides such as C5 (residues 96–123), C6 (residues 119–152), C9 (residues 205–233) or C11 (residues 255–280) can induce significant proliferation of T cells in short-term cultured T cell lines from some RV-seropositive donors (Ou et al., 1992). The T cell clones used in this study were initially established from the T cell lines of two RV seropositive donors (Ou et al., 1992). A total of 36 clones were isolated by the method

TABLE 1

REACTIVITY OF RUBELLA SPECIFIC T-CELL CLONES TO SYNTHETIC PEPTIDES OF C PROTEIN

Synthetic peptide	Amino acid position	Cell proliferation of T cell clone (cpm)[a]						
		R10	R11	R18	A2	A10	A11	A12
C1	1–30	176	97	210	156	155	238	207
C2	28–56	218	199	208	204	185	242	162
C3	52–78	204	127	305	187	158	195	198
C4	74–100	191	234	408	174	147	113	212
C5	96–123	302	204	246	265	173	205	114
C6	119–152	9384	18597	5958	302	242	400	103
C7	151–179	184	215	248	155	374	207	157
C8	177–204	262	217	362	189	254	130	111
C9	205–233	223	312	262	7166	238	8704	145
C10	231–257	325	182	324	223	242	149	244
C11	255–280	324	265	567	308	6598	148	5532[b]
None		270	263	256	164	310	190	209

[a] A total of 2 × 10⁴ T cells from each clone was tested for their proliferative response to each synthetic peptide at a final concentration of 5 μg/ml in the presence of γ-irradiated (3000 rad) autologous PBMC 5 × 10⁴ as APC. Results are presented as the mean cpm obtained from triplicate determinations.

[b] Underlined numbers represent significant cell proliferation values.

of limiting dilution (Celis et al., 1988a,b) and their RV specificity was tested in proliferation assays (Ou et al., 1992). To further identify the capacity of these T cell clones to recognize the T cell epitopes of the structural proteins of RV, their responses to 49 synthetic peptides were tested in proliferation assays. Based on their positive responses to both UV-inactivated RV and individual C peptides, 7 clones were selected for this study. T cell clones (R10, R11, and R18) isolated from an oriental donor (RM) reacted strongly only with peptide C6 (residues 119–152) (Table 1). T cell clones A2, A10, A11, and A12 were isolated from a Caucasian donor (AT). Clones A2 and A11 responded to C9 (residues 205–233), while clones A10 and A12 reacted with C11 (residues 255–280) (Table 1). Surface marker analysis of all T cell clones showed that more than 99% of cells were CD3+, CD4+, and CD8⁻ cells (data not shown), demonstrating that these T cell clones are probably T helper cells.

The cytotoxic activities of capsid peptide-specific T cell clones were studied by using autologous Epstein-Barr virus-transformed B cell line (EBV-BL) cells as targets in a standard ⁵¹Cr release assays (Celis et al., 1988a). Strong and specific cytotoxic responses of T cell clones R11, R18, A2, A10, A11, and A12 were observed when target cells were preincubated with either RV or peptides C6, C9, or C11, respectively (Fig. 1). The results indicated that T cell clones R11 and R18 had the ability to kill autologous EBV-BL target cells incubated with peptide C6 and that T cell clones A2 and A11, A10, and A12 had cytotoxic activities to autologous EBV-BL cells expressing peptide C9 or C11 de-

terminants. Target cells that were incubated with medium alone or with UV-inactivated EBV or other peptides of RV were not killed by these T cells or were only marginally lysed in a dose-independent manner (data not shown), indicating that the cytotoxic responses were indeed Ag-specific.

In order to identify the minimal antigenic peptide for these T cell clones, a nested set of truncated peptides within the relevant region of C6, C9 or C11 determinant were prepared and tested, in both proliferation and ⁵¹Cr release assays. Peptides C6B (residues 119–145), C6C (residues 124–145) and C6E (residues 125–139) were equally efficient in proliferation assays, as compared to the antigenic 34-mer peptide C6 (C6A, residues 119–152). C6E (15 residues) is the shortest peptide that could stimulate T cell clones R11 and R18 (Table 2). The results obtained in cytotoxicity assays with T cell clones R10, R11 and R18 were found paralleled with the results in proliferation assays (data not shown). Thus these T cell clones displayed cytotoxic activities against autologous EBV-BL targets expressing epitopes C6C and C6E but not C6D, C6F or C6H. Besides the C9 peptide (C9A, residues 205–233), only the truncated peptide C9B (residues 205–216) stimulated the proliferative responses of T cell clones A2 and A11 (Table 2). Peptides C9C (residues 210–221), C9D (residues 212–223), C9E (residues 222–233), and C9F (residues 216–227) gave negative results (Table 2). Cytotoxic responses of A2 and A11 clones to truncated C9 peptides were found similar to the proliferation responses (data not shown). T cell clones A10 and A12 responded to all C11 peptides, the antigenic 26-
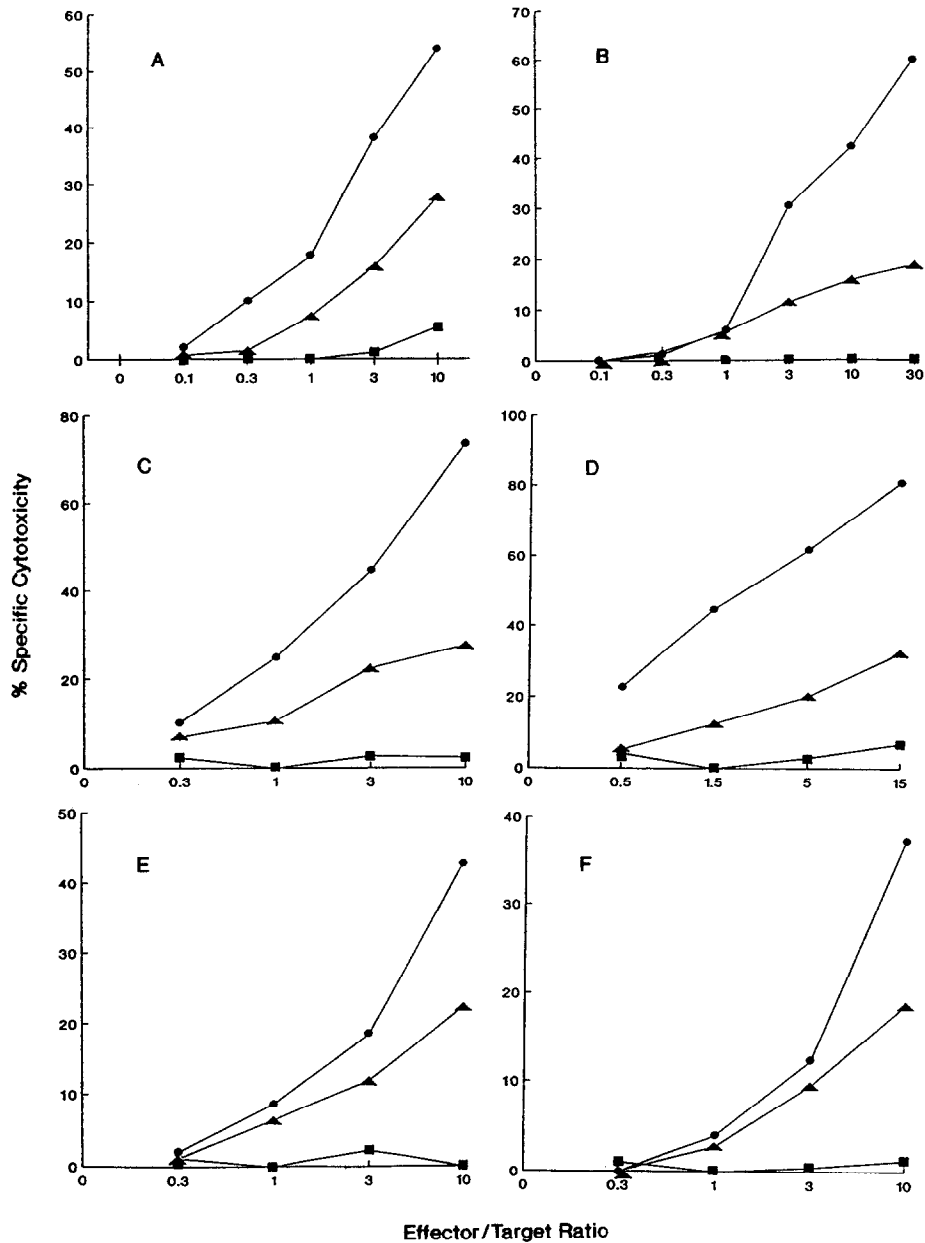
FIG. 1. Cytotoxic activity of T cell clones. CTL responses of six C peptide-specific T cell clones were tested in a standard ^{51}Cr release assay. Target cells used were autologous EBV-BL cells incubated with corresponding C peptide at 5 μg/ml (●) or UV-inactivated RV at 1 × 10^6 FFU/ml overnight before addition of T cells (▲) or EBV-BL cell alone (■). T cell clones and corresponding specific peptides are (A) R11-C6, (B) R18-C6, (C) A2-C9, (D) A11-C9, (E) A10-C11, and (F) A12-C11.

mer C11 peptide (C11A, residues 255–280) as well as truncated C11B (residues 255–276), C11C (residues 260–276), C11D (residues 264–276), C11E (residues 260–272), and C11F (residues 264–272) in proliferation assays (Table 2). C11F is the shortest C11-truncated peptide that stimulated the T cell clones with significant cell proliferation responses (Table 2). In ^{51}Cr release assays, T cell clones A10 and A12 were cytotoxic against autologous EBV-BL target cells expressing peptides C11 (C11A), C11B, C11C, and C11E, but

not C11D and C11F (data not shown). These results suggest that there is some difference in the T cell recognition of C11 epitope between two T cell functional assays. It appears that the induction of cytotoxic responses of these T cell clones to C11-binding targets requires residues TTER of C11 sequence.

We are interested in determining whether these T cell clones were restricted through different HLA determinants. mAbs specific to monomorphic regions of the HLA-A.B.C., HLA-DR, and HLA-DQ antigens were

TABLE 2

Antigenicity of Truncated Peptides for T Cell Clones

Peptide	Positions	Amino Acid Sequence[b]	Cell Proliferation (cpm)[a]	
			(R11)	(R18)
C6A	119-152	PELGPPTNPFQAAVARGLRPPLHDPDTEAPTEAC	4172	3036
C6B	119-145	PELGPPTNPFQAAVARGLRPPLHDPDT	4350	4428
C6C	124-145	PTNPFQAAVARGLRPPLHDPDT	5275	4173
C6D	131-145	AVARGLRPPLHDPDT	485	568
C6E	125-139	TNPFQAAVARGLRPP[c]	3449	4114
C6F	128-139	FQAAVARGLRPP	515	568
C6G	119-133	PELGPPTNPFQAAVA	983	808
C6H	125-133	TNPFQAAVA	400	465
none			468	409
			(A2)	(A11)
C9A	205-233	VRAYNQPAGDVRGVWGKGERTYAEQDFRV	7394	8441
C9B	205-216	VRAYNQPAGDV	3937	2622
C9C	210-221	QPAGDVRGVWGK	577	645
C9D	212-223	AGDVRGVWGKGE	288	319
C9E	222-233	GERTYAEQDFRV	296	419
C9F	216-227	RGVWGKGERTYA	303	309
none			216	240
			(A10)	(A12)
C11A	255-280	PLPPHTTERIETRSARHPWRIRFGAP	4065	3296
C11B	255-276	PLPPHTTERIETRSARHPWRIR	4102	3973
C11C	260-276	TTERIETRSARHPWRIR	4511	3122
C11D	264-276	IETRSARHPWRIR	7392	4045
C11E	260-272	TTERIETRSARHP	6099	6968
C11F	264-272	IETRSARHP	6253	1632
none			260	413

[a] A total of 2 × 10⁴ T cell from each clone were tested for their proliferative response to each synthetic peptide at a final concentration of 1 μg/ml in the presence of APC (6000-rad γ-irradiated autologous EBV-BC cell).

[b] The amino acid sequence of each peptide was derived from the nucleotide sequence corresponding to the C protein of RV M33.

[c] Underlined sequence indicates the shortest peptide with significant cell proliferation. Responsor T cell clones are shown in parentheses.

tested for their ability to block presentation of the C6 and C11 peptide epitopes by autologous peripheral blood mononuclear cells (PBMC). Stimulation of clones R10 and R11 by peptide C6, clone A11 by peptide C9 and clone A12 by peptide C11 were clearly inhibited in the presence of anti-HLA-DR, DQ mAb 9.3F10 (Van Voorhis *et al.*, 1983) and anti-HLA-DR mAb L243 (Lampson and Levy, 1980), but not in the presence of the anti-HLA-A.B.C. mAb W6/32 (Brodsky and Parham, 1982) and anti-HLA-DQ mAbs IVD12 (Giles *et al.*, 1983) (data not shown). These results indicate that these T cell clones were HLA-DR-restricted. In order to complete a detailed analysis of the genetic restriction of the capsid peptide specific T cell clones, we used 10 EBV-BLs with different HLA phenotypes as APCs in proliferation assays (Table 3) and as targets in cytotoxicity assays. Six homozygous human HLA phenotype cell line (HCL) cells were used as target cells in ⁵¹Cr release assays in this study (Table 4). The characterization of the restriction elements of T cell clones R11 and R18 was established by using both proliferation and ⁵¹Cr release assays. The peptide C6 (residues 119–

152) was presented by EBV-BL RM and DO, but not by others in proliferation assays (Table 3). EBV-BL RM is an autologous B cell line of T cell clones R11 and R18. The most important difference between EBV-BL DO and 6 other EBV-BLs is that the former shares the HLA DRw9 phenotype with RM (Table 3). Similar results were observed in ⁵¹Cr release assays. Five EBV-BLs were used as target cells. These T cell clones recognized only RM and DO targets but not HE, MS, and AT targets (data not shown). These results suggested that the restriction molecule used to present the C6 determinant is of DRw9-associated haplotype.

In order to define the restriction molecules in the responses of T cell clone A11 to the C9 determinant, we tested the cytotoxic responses of T cell clone A11 against six HCLs and five EBV-BLs previously incubated with the C9 peptide. The results demonstrated that T cell clone A11 recognized only HCL PRIESS (Table 4) and EBV-BLs CM, AT, and MT (data not shown), all of which share the HLA DR4 phenotype. EBV-BL AT is the autologous EBV-BL of T cell clone A11. The results of proliferation assays using EBV-BLs as APCs correlated well with the results of these cytotoxicity assays. T cell clones A2 and A11 responded to C9 peptide when this peptide was presented by EBV-BLs (CM, HC, MS, AJ, AT, and MT) which expressed the DR4 phenotype, but not by RM and LM (data not shown). Hence, DR4 is the restriction element presenting the C9 peptide epitope to T cell clone A11.

The restriction specificity of T cell clones A10 and A12 to the C11 determinant was analyzed by using ⁵¹Cr release assays. Six HCLs and EBV-BL AT were used as targets for T cell clone A12. The results show that T cell clone A12 was strongly lytic only against EBV-BL AT and HCL PRIESS, which share the HLA DR4 phenotype with the autologous EBV-BL AT (Fig. 2A). When clone A10 was used as the effector, four EBV-BL cells previously incubated with C11 peptide were used as target cells in cytotoxicity assay. The results indicated that the T cell clone A10 was cytotoxic against targets (EBV-BLs CM, AT, and MT) which expressed the DR4 phenotype, but not against target RM (Fig. 2C). These results demonstrate that the genetic restriction molecule of T cell clones A10 and A12 is DR4. In proliferation responses of T cell clones A10 and A12, however, EBV-BL LM, whose DR phenotype is DR1, like CM, HC, MS, AJ, and AT, were able to present the C11 determinant (data not shown). In order to further test the ability of EBV-BL LM presenting C11 to T cell clone A12, we used EBV-BL LM cells as the targets in the ⁵¹Cr release assay. The results indicated that EBV-BL LM was able to elicit a cytotoxic response of T cell clone A12 to peptide C11 similar to that elicited by target cells such as AT and AJ (Fig. 2B).

TABLE 3

HLA Class II Restriction of C6-Specific T Cell Clone[a]

APC	HLA phenotype of APC[b]					Cell proliferation (cpm) T cell clone	
	A	B	C	DR	DQ	R11	R18
RM	2,11	13,16 w4, w6	w3	w9 w53	w3	29,982	15,719[c]
CM	1, 32	44, 35 w6	w4	1, 4 w53	w1, w3	931	318
DO	2	60 w6		w9 w53	w3	10,782	11,889
HC	24, 30	14, 46 w6	w1, w3	4, w8 w52, w53	w4	228	492
MS	2, 24	18, 62 w6	w3	4, w11 w52, w53	w7, w8	187	528
LM	2	35 w6		1, w13 w52	w1	185	329
AJ	1, 29	8, 45 w6	w6	4, w17 w52, w53	w2, w7	151	408
RD	2, 19	14, 49 w4, w6	w7	4 w53	w3	223	383
AT	3, 11	51, 57 w4	w3, w6	4, 7 w53	w3	ND	ND
MT	2, 3	51, 50 w4, w6	w3	4 w53	w3 (7)	ND	ND

[a] Cloned T cells 2 × 10⁴ were cultured for 72 hr in the presence of γ-irradiated (3000 rad) EBV-BL cells 2 × 10⁴ as APC from 10 HLA-typed donors with or without C6 peptide (5 μg/ml). [³H]thymidine was added over the last 18 hr of culture. Results are expressed as the mean cpm of triplicate cultures.

[b] APC RM is autologous EBV-BL for these C6-specific clones.

[c] All cpms from the culture without C peptide were under 500 cpm. ND, not tested.

Two models have been proposed to predict and identify the amino acid sequences of antigen involved in T cell recognition. De Lisi and Berzofsky (1985) have proposed that a large proportion of lymphocytes appear to react with α-helical amphipathic structures (AS) contained within the amino acid sequences of the antigen proteins (Margalit et al., 1987). On the other hand, the study by Rothbard and Taylor (1988) points to the existence of a 4- to 5-amino acid sequence motif in a large number of T cell antigenic determinants. Consistent with the first model, we found that C6E, C9B, and C11E, which were efficient in inducing both proliferative and cytotoxic responses in the corresponding C6, C9, or C11-reactive T cell clones, are located at the

TABLE 4

Specific Cytotoxicity of T Cell Clone A11 to Targets with Different HLA-DR Phenotype While Targets Expressed C9 Determinant

Cell line	HLA Phenotype				Percentage specific ⁵¹Cr release[a]
	A	B	C	DR	
MAjA	2, 2	35, 35		1, 1	1.6[b]
MST	3, 3	7, 7		2, 2	3.2
WT-20	30, 30	18, 18		3, 3	3.0
PRIESS	2, 2	15, 15	6, 6	4, 4	71.6
Lg-38	25, 25	18, 18		5, 5	0
MANN	29, 29	12, 12		7, 7	5.1

[a] Cell lines used as targets were incubated with or without peptide C9 at 5 μg/ml overnight before addition of T cells.

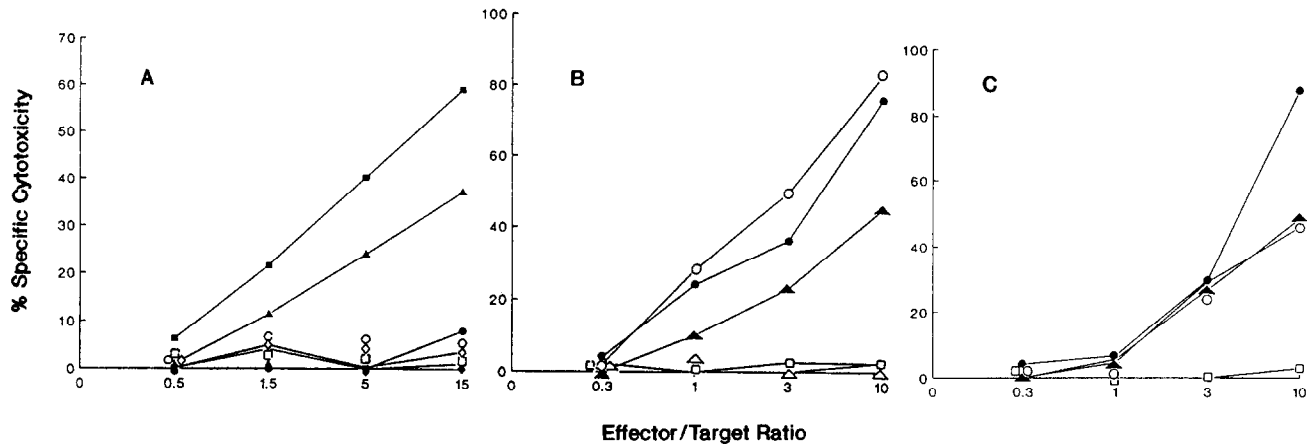[b] Cytotoxicity was determined using the T cell clone A11 at an effector/target ratio of 5.

FIG. 2. Cytolytic activity of T cell clone A12 and A10 to C11 determinant presented on target cells with different HLA phenotypes. (A) Clone A12 is effector. Target cells used were human HLA homozygous cell lines MAJA (□), MST (O), WT-20 (●), PRIESS (■), Lg-38 (◇), MANN (◆), and EBV-BL AT (▲). (B) Clone A12 is effector. Target cells used were EBV-BL RM (□), DO (△), LM (▲), AJ (O), and AT (●). (C) Effector is clone A10. Target cells used were EBV-BL RM (□), CM (O), AT (●), and MT (▲). AT is autologous EBV-BL. Target cells were incubated with C11 peptide (5 μg/ml) overnight before addition of T cells.

peak region of the α-helix index within the C6, C9, or C11 peptide sequences according to the profiles of α-helix, β-turn and hydrophilicity of the C protein of RV (Ou *et al.,* 1992). We have analyzed the T cell motif (second model) structures within C6, C9, and C11 sequences (Ou *et al.,* 1992). C6E is located in the region between two T cell motif structures. C9B contains a 4-amino acid T cell motif structure. The C11E sequence contains only half of such a 4-amino acid structure. Although antigenicity prediction methods are popular for predicting the location of viral epitopes, our results indicate that that latter model may not achieve a high level of correct prediction.

The questions of HLA class II polymorphism and epitope selection are clearly important issues in the design of subunit or synthetic vaccines for the genetically diverse human populations. The HLA restriction patterns of T cell clones A10 and A12 are more complex than those of the clones mentioned above. Besides six EBV-BLs of HLA-DR4 phenotype, EBV-BL LM of DR1 phenotype could also present peptide C11 to stimulated clones A10 and A12 in proliferation responses (data not shown). Similar genetic restriction by HLA DR4 was shown in cytotoxicity assays (Fig. 2). Interestingly, T cell clone A12 was cytotoxic against targets AJ, AT, MT (DR4 phenotype), and LM (DR1 phenotype), which presented C11 determinants (Fig. 2B). These results suggest that there is some cross-recognition by T cell clones between DR1 and DR4 subtypes. This phenomenon is consistent with reports regarding HLA restriction of human T cell clones (Rees *et al.,* 1989; Lombardi *et al.,* 1989; Lamb *et al.,* 1988). However, in contrast to this, T cell clone A12 exhibited specific cytotoxic responses against HCL PRIESS (DR4 pheno-

type), which expressed C11, but not against HCL MAJA of the HLA-DR1 phenotype (Fig. 2A). There may be differences in the structure of the antigen-binding site between the DR1 molecules of HCL MAJA and EBV-BL LM. Three different DR1 subtypes that have amino acid substitution in the putative peptide-binding site (residues 52–86 of β domain) have been identified (Marsh and Bodmer, 1989). These differences between DR1 subtypes may result in DR4-restricted C11 peptide not binding with sufficient affinity or in the required conformation of the DR1 molecule of HCL MAJA to stimulate the T cell clone. There is no cross-recognition by T cell clones A2 and A11 between DR1 and DR4 subtypes. Similar results have been reported in the study of human T cell recognition of influenza virus (Brett *et al.,* 1991).

Examinations of the fine specificity of T cell recognition of the protein antigens of viruses and parasites in both human and murine systems have revealed multiple but distinct T cell sites among individuals with different HLA phenotypes or a panel of H-2 congenic strains. Due to the limits imposed on T cell antigen recognition by MHC restriction, it is becoming apparent that a number of T cell sites will be required to ensure that a synthetic vaccine will engage the majority of an outbred population. Presentation of a number of short synthetic T cell sites within a particular structure may be an economical and feasible way to accomplish this task (Milich, 1988). The recognitions of C9 and C11 by T cell clones were associated with DR4, which was the most frequently occurring HLA DR allele among orientals (22%) and is also common among Caucasians (13%). Furthermore, HLA-DRw9 is also frequent among orientals (12%) (Giles and Capra,

1985). Recently we have tested 20 T cell lines from 10 RV patients and 10 healthy donors with different HLA phenotypes by using 11 synthetic C peptides. Our results demonstrate that C11, C6, and C9 are recognized by these T cell lines at a higher frequency than other peptides (unpublished data). These T cell determinants may be selected as the candidates for T cell sites for future RV vaccine development.

ACKNOWLEDGMENTS

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