SPECIFIC KILLING OF CYTOTOXIC T CELLS AND ANTIGEN-PRESENTING CELLS BY CD4+ CYTOTOXIC T CELL CLONES

A Novel Potentially Immunoregulatory T-T Cell Interaction in Man

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Resistance to intracellular microorganisms such as mycobacteria is dependent on antigen-specific MHC class II-restricted helper T cells (Th) (1). Th cells are thought to release macrophage-activating factors that enable macrophages to eliminate the infectious organism (1). It has recently become clear, however, that exposure to intracellular parasites not only induces a Th cell but also a CTL response. CTL may be important effector cells in the protective immune response to these parasites (reviewed in reference 2). For example, *Mycobacterium leprae* and *M. bovis* BCG are potent inducers of antigen-specific HLA-restricted CTL that lyse human macrophages (3, 4). These CTL can belong to both the $CD8^+$ and the $CD4^+$ subset, and are readily detectable after primary in vitro exposure of PBMC to antigen (3, 4, Birhane Kale Ab, R. Kiessling, P. Converse, E. Halapi, G. Tadesse, and T. H. M. Ottenhoff, manuscript submitted for publication). We have also shown that the 65-kD mycobacterial heat shock protein (hsp)[1] is an important target antigen for polyclonal $CD4^+$ HLA-DR-restricted CTL (3, 5).

CTL lyse target cells that express the ligand for the TCR, i.e., a peptide fragment of antigen complexed to an MHC molecule (6, 7). Such antigen fragments are produced by the processing machinery of the target cell, and can often be replaced by synthetic peptides (7). Thus, target cells become susceptible to CTL-mediated lysis upon incubation with exogenous, "preprocessed" peptide antigen in vitro. CTL lyse target cells through a complex mechanism, that may involve the release of cytotoxic granules that contain lytic effector molecules like perforin, serine esterases, and various other mediators, but also granule exocytosis-independent lytic pathways have been described (reviewed in references 8, 9). In either case, however, CTL were found to be remarkably resistant against lysis by themselves (8–10), by other primary (11) or cloned (8–10, 12–15) CTL, by purified granules (11, 14–16), and by purified perforin itself (17, 18). Most studies addressing this CTL resistance to lysis have been performed with mouse $CD8^+$ CTL.

This study was supported by the Dutch Organization for Scientific Research, by the Immunology of Leprosy Component of the World Health Organization and by the European Community.

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[1] *Abbreviations used in this paper:* CS, circumsporozoite antigen; hsp, heat shock protein.

We have now further characterized the CTL response against the mycobacterial 65-kD hsp. We found that the mycobacterial 65-kD hsp triggers $CD4^+$ CTL clones that are specific for different peptide defined epitopes (19, 20). These CTL not only lyse APCs but also $CD4^+$ or $CD8^+$ CTL clones, including themselves, that carry the proper peptide/HLA class II complex. $CD4^+$ CTL are therefore not by definition resistant to CTL- or self-mediated lysis; in addition, CTL-mediated CTL lysis may represent a novel immunoregulatory pathway in man, since human T cells, in contrast to murine, express MHC class II.

Materials and Methods

Antigens. The recombinant 65-kD hsp of *M. bovis* BCG was a gift of Dr. J. D. A. van Embden (Bilthoven, The Netherlands). Peptides of the *M. leprae* 65-kD hsp were made by solid-phase peptide synthesis and checked by analytical reverse-phase HPLC and amino acid analysis as described previously (20), and were a kind gift of Dr. D. C. Anderson (Seattle, WA). The *Plasmodium falciparum* CS peptide was a gift of Dr. F. Sinigaglia (Basel, Switzerland) (21). The sequences of the peptides are given in Table I.

T Cell Clones. The T cell clones used in this study are summarized in Table I. Some of them have been described previously with regard to their antigen specificity and HLA class II restriction (19, 20, 22–24).

Cytotoxicity Assay. After overnight pulsing with antigen (5 μg/ml), target cells were pelleted, resuspended, and labeled with 100 μCi ^{51}Cr sodium chromate (Amersham International, Amersham, UK) during 1 h at 37°C. After three washes, target cells were added to 96-well round-bottomed plates (Costar 3799) in 100 μl of medium at 10^4 cells/well in triplicate wells. Effector cells were then added in 100 μl at the indicated E/T cell ratios. The plates were incubated for 4 h and then centrifuged, after which cell-free supernatants were collected from each well, and ^{51}Cr release was counted in a gamma counter. The percent specific lysis was calculated using the following formula: percent specific lysis = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100%. All cultures were set up in Iscove's modified Dulbecco's Medium (Gibco Laboratories, Grand Island, NY) supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml) (both Gibco Laboratories, Grand Island, NY) and 10% pooled human AB serum.

Results

65-kD hsp or CS Peptide-dependent Lysis of EBV-BLCL Targets by $CD4^+$ Cytotoxic T Cell Clones. Peptide pulsed or nonpulsed HLA class II matched EBV-BLCL cells were used as target cells for freshly cultured $CD4^+$ HLA class II-restricted T cell clones that recognized specific peptides of the mycobacterial 65-kD hsp (see Table I). As shown in Fig. 1, all clones strongly lysed target cells pulsed with the specific peptide but not targets pulsed with irrelevant or no peptide. A control *P. falciparum* CS peptide-specific $CD4^+$ T cell clone, MGD5, was included that gave similar results. Thus, T cell clones that recognize different peptides in combination with DR or DO restriction elements and that are derived from different individuals, are cytotoxic for peptide-pulsed target cells.

Clones N1C8 and W2G2 were able to lyse EBV-BLCL that had been pulsed with the purified, whole recombinant 65-kD hsp. Clone R1F9 showed minimal lysis and the other clones no lysis at all of recombinant 65-kD hsp pulsed EBV-BLCL. Since these latter clones are able to lyse macrophages under similar conditions (data not shown), these results point to a relative presentation defect of these EBV-BLCL to some of the 65-kD hsp-specific T cell clones. This defect is apparently overcome when peptide is used instead of the recombinant 65-kD molecule.

TABLE I

T Cell Clones and the Amino Acid Sequences of the M. leprae 65-kD hsp and P. falciparum CS Peptides Used in This Study

Pathogen	Protein	Position	Sequence (one-letter amino acid code)	Recognized by T cell clone	Restricted by
M. leprae	65-kD hsp	3-28	KTIAYDEEARRGLERGLNSLADAVKV	R1F9	HLA-DR3
		3-13	KTIAYDEEARR	R1F9	HLA-DR3
		183-204	LQLELTEGMRFDKGYISGYFVT	R3F10	HLA-DQ
		412-425	GGGVTLLQAAPALD	W2G2, N3A9, N1C8	HLA-DR1
		416-439	TLLQAAPALDKLKLTGDEATGANI	R2F10, RP_{171}-2	HLA-DR2
		418-427	LQAAPALDK	R2F10, RP_{171}-2	HLA-DR2
P. falciparum	CS	378-398	DIEKKIAKMEKASSVFNVVNS	MGD5	HLA-DR2

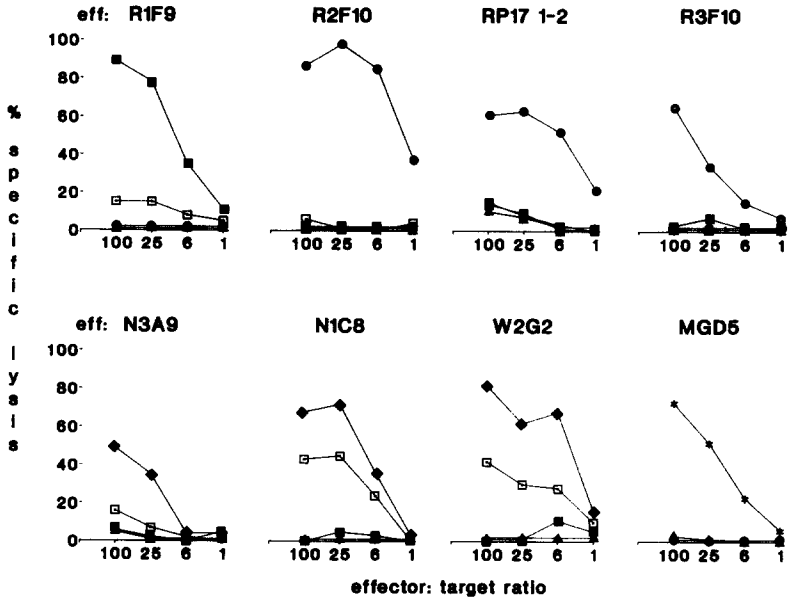

FIGURE 1. 65-kD hsp or CS peptide-specific lysis of EBV-BLCL targets by CD4+ cytotoxic T cell clones. CD4+ effector T cell clones were added to target cells at the indicated E/T ratios. Target cells were HLA class II matched or autologous EBV- BLCL (10^4 cells/well) that had been pulsed with antigen during 18 h before the 4-h ^{51}Cr-release assay. Antigens are indicated by the following symbols: (●) 416- 439 peptide of 65-kD hsp, (○) 183-204 peptide of 65-kD hsp, (■) 3-28 peptide of 65-kD hsp, (◆) 412-425 peptide of 65-kD hsp, (⊡) r65kD hsp, (*) 378-398 peptide of CS, (▲) no antigen.

One representative experiment (Table II) shows that the level of target cell lysis is dependent on the peptide concentration during the pulsing phase. This further underlines the antigen dependency and specificity of target cell lysis by CD4+ clones.

CD4+ 65-kD hsp-specific CTL Lyse Themselves and Other CD4+ CTL in the Presence of Specific Peptide. Human T cell clones, in contrast to mouse T cells, strongly express MHC class II antigens. Synthetic peptides can bind directly to MHC class II. Since CTL usually are resistant to lysis by self- or other CTL or subcellular CTL components, we investigated if peptide pulsed CTL would be susceptible to CTL-mediated lysis. The results in Table III, A and B show that all 65-kD hsp-specific CTL clones lyse themselves as well as other autologous T cell clones that had been pulsed with relevant peptide. Both TCR-α/β and TCR-γ/δ cells (data not shown) are susceptible to lysis. In contrast, an HLA mismatched clone (SCL 5.12, Table IIIA) is not lysed.

Although EBV-BLCL can present (probably processed) native recombinant 65-kD hsp to some T cell clones (W2G2, N1C8; Table IIIB), T cell clones are unable to do so (Table IIIB).

So far, all tested 65-kD hsp peptide-specific CD4+ T cell clones are able to lyse CD4+ CTL in the presence of appropriate peptide. Thus, our results show that peptide-specific CD4+ CTL are able to lyse themselves as well as other CD4+ CTL in the presence of specific peptide.

TABLE II
Peptide Concentration Dependency of Target Cell Lysis

Target cell	Pulsed with 65-kD hsp peptide	Effector: R1F9 E/T: 50:1	13:1	3:1	1:1
EBV-BLCL	–	0	0	0	0
	3-12 0.005 μg/ml	9	4	3	0
	3-12 0.05 μg/ml	21	11	3	0
	3-12 0.5 μg/ml	39	33	19	16
	3-12 5.0 μg/ml	73	56	23	6
	418-427 5.0 μg/ml	0	0	1	1

Target cells were incubated with the indicated peptide concentrations for 18 h before labeling with ^{51}Cr. Assays were set up in triplicate with 10^4 target cells per well. Effector cells were added to wells at the indicated E/T cell ratios. ^{51}Cr release in culture supernatants was determined after 4 h. Results are expressed as percent specific lysis.

$CD4^+$ 65-kD hsp Peptide-specific CTL Lyse HLA Class II Compatible $CD8^+$ CTL Clones in the Presence of Specific Peptide. Since most studies addressing the resistance of CTL against self-mediated killing have focused on the "classical" $CD8^+$ CTL, we tested four randomly selected HLA class II compatible $CD8^+$ CTL clones, specific for HLA class I/minor transplantation antigens (6, 25, 26). CTL C754.5w4 could only be lysed by effector CTL R2F10 but not R1F9, as expected on the basis of its HLA DR phenotype (Table IV). The other three $CD8^+$ CTL clones could be lysed by both $CD4^+$ CTL clones, provided that they had been pulsed with the proper 65-kD peptide. The levels of lysis were much lower that those seen with EBV-BLCL targets, suggesting a partial but not total protection from CTL-mediated lysis. Thus, peptide-pulsed human $CD8^+$ cytolytic CTL clones can be lysed by $CD4^+$ CTL clones, and therefore are not absolutely resistant against CTL-mediated lysis.

$CD4^+$ CTL-mediated Lysis Does Not Induce Nonspecific Bystander Lysis. $CD4^+$ T cell clones have been reported to acquire nonspecific lytic activity upon long-term culture in IL-2. Although we found no evidence for nonspecific lysis, we tested whether the presence of a high dose of rIL-2 in the assay could trigger such nonspecific lysis. Lysis remained completely specific in the presence of excess rIL-2 (100 U/ml) or after overnight preincubation of target cells with an excess rIFN-γ (250 U/ml) (data not shown).

In Table V, subsequent experiments are shown in which unlabeled (cold) antigen-pulsed targets were mixed with ^{51}Cr-labeled (hot) unpulsed targets in order to exclude nonspecific bystander lysis. The three experiments show that CTL strongly lyse hot, peptide-pulsed EBV-BLCL. When peptide pulsed targets were added as cold targets to nonpulsed hot targets, the lysis of the former did not induce significant lysis of the latter, bystander targets. Three cold targets were tested: EBV-BLCL, K562 (a human NK target), and a CTL clone. Other experiments showed that killing was an active, temperature- and contact-dependent process, including Ca^{2+} influx in CTL immediately upon activation by target cells (not shown). Thus, the effector phase of lysis by $CD4^+$ CTL is entirely specific and does not induce bystander lysis.

Cold Target Competition Experiments. To further confirm the specificity of lysis, cold target competition experiments were carried out. Fig. 2 gives one representative ex-

TABLE III

Antigen-specific Lysis of CD4+ T Cells by CD4+ CTL Reactive with 65-kD hsp of Mycobacteria

A. Target cell	Pulsed with 65-kD hsp peptide	Effector cells: R1F9 E/T: 100:1	25:1	6:1	R2F10 100:1	25:1	6:1	RP_{171}-2 100:1	25:1	6:1
EBV-BLCL	3-28	89	78	35	0	0	0	15	8	3
	416-439	0	0	3	87	98	85	61	63	52
	–	0	0	0	1	0	0	10	7	2
R1F9	3-28	82	50	42	0	0	0	ND	ND	ND
	416-439	0	0	0	90	78	55	58	77	64
	–	0	0	0	0	0	0	0	0	0
R2F10	3-28	68	60	50	4	0	2	0	0	2
	416-439	5	11	0	43	60	49	58	48	25
	–	0	0	0	0	0	0	0	0	0
RP_{171}-2	3-28	84	88	49	0	0	0	0	0	2
	416-439	4	0	0	76	72	27	44	22	13
	–	4	2	0	0	0	0	5	3	1
SCL5.12*	3-28	3	4	1	0	0	0	0	0	1
	416-439	0	0	0	0	0	0	0	0	0
	–	3	2	0	0	0	0	0	2	0

B.		Effector cells: W2G2 E/T: 100:1	25:1	6:1	N1C8 100:1	25:1	6:1	N3A9 100:1	25:1	6:1
EBV-BLCL	r65 kD hsp	42	30	28	43	45	24	16	7	2
	412-425	82	62	68	67	72	36	49	35	4
	–	0	0	1	0	5	4	5	0	0
W2G2	r65 kD hsp	ND	ND	ND	ND	ND	ND	ND	ND	ND
	412-425	60	80	53	72	72	21	31	24	3
	–	0	7	0	6	0	0	0	0	0
N1C8	r65 kD hsp	0	0	0	5	5	0	0	0	0
	412-425	27	36	24	32	22	13	21	21	0
	–	0	2	3	1	0	0	3	0	0
N3A9	r65 kD hsp	0	0	0	0	0	0	0	0	0
	412-425	50	35	59	35	42	35	21	32	36
	–	0	6	6	0	0	0	4	7	5

		Effector: R3F10 E/T: 100:1	25:1	6:1
EBV-BLCL	3-28	3	7	0
	183-204	65	34	15
	–	0	0	0
R3F10	3-28	0	0	0
	183-204	51	37	0
	–	0	0	0
RP_{151}-1	3-28	0	0	0
	183-204	39	35	0
	–	0	0	0

Target cells were incubated with 5 μg/ml of the indicated antigens for 18 h before labeling with ^{51}Cr (see footnote to Table II). The results are expressed as percent specific release.

* HLA mismatched clone.

TABLE IV
Lysis of CD8⁺ CTL Clones by CD4⁺ CTL Clones

Target cell	HLA-DR type	Pulsed with 65-kD hsp peptide	CD4⁺ effector cells					
			R1F9			R2F10		
		E/T:	100:1	25:1	6:1	100:1	25:1	6:1
EBV-BLCL	2,3	3-28	88	64	24	0	6	6
		416-439	2	0	3	86	64	53
		–	4	2	0	3	5	3
CD8⁺ CTL		3-28	1	2	2	0	1	1
Wam C754.SW4	1,2	416-439	0	0	1	24	20	13
		–	0	0	0	0	0	2
CD8⁺ CTL		3-28	23	33	19	0	0	7
Hel 1E6	2,3	416-439	0	3	3	29	23	20
		–	1	5	5	0	5	8
CD8⁺ CTL		3-28	11	13	10	0	2	6
Hel 5H4	2,3	416-439	1	0	3	28	36	15
		–	2	2	4	1	0	4
CD8⁺ CTL		3-28	36	15	12	0	5	6
Hel 1E4	2,3	416-439	0	2	6	28	25	38
–	–	–	0	0	0	0	0	0

Target cells were pulsed overnight with 5 μg/ml of the indicated peptides. Assays were set up in triplicate with 10^4 target cells per well. Effector cells were added to wells at the indicated E/T cell ratios. ^{51}Cr release in culture supernatants was determined after 4 h. Results are expressed as percent specific lysis.

ample. Lysis of peptide-pulsed EBV-BLCL could be competed away in a dose-dependent fashion by cold EBV-BLCL that had been pulsed with the same peptide (Fig. 2 *A*) but not by the nonpulsed cold target (Fig. 2 *B*). Peptide-pulsed but not unpulsed EBV-BLCL were very efficient in competing away lysis of peptide pulsed CTL targets: (Fig. 2, *C* and *D*). When peptide-pulsed or nonpulsed CTL were used as cold competitors for peptide-pulsed EBV-BLCL, a very high spontaneous release was observed because, as expected, these cold CTL efficiently lysed peptide pulsed EBV-BLCL (data not shown).

Similar data were obtained for another CTL clone. Thus, although CD4⁺ CTL efficiently lyse peptide-pulsed CTL targets, they bind more efficiently to EBV-BLCL targets. This may also explain the often lower levels of lysis that are observed with CTL as compared with EBV-BLCL targets (see Table III, *A* and *B*).

Effect of Target Cell or Effector Cell Prepulsing with Peptide. We next tried to replace the prepulsing of target cells with peptide by adding free peptide into the assay. Even very high peptide concentration (up to 100 μg/ml) gave suboptimal lysis as compared with lysis of targets that had been pulsed overnight (5 μg/ml) (not shown). This was unexpected since in other studies, the addition of free (tetanus toxoid) peptide to EBV-BLCL led to an almost immediate Ca^{2+} influx in an antigen-specific CD4⁺ T cell clone (27). Besides considering other explanations (the slow HLA-peptide complex formation [28]; the higher complexity of target cell lysis as compared with merely TCR/CD3 signaling; the induction of peptide-dependent self-mediated CTL killing in the presence of the free peptide), we tested whether the direct binding of free peptide to the TCR possibly interfered with (or led to) target

TABLE V

CD4+ CTL Lyse Target Cells in the Absence of Bystander Lysis

Exp.	Hot target pulsed with 65-kd hsp peptide	Cold target + pulsed with 65-kD hsp peptide	Effector cells								
			R2F10			RP_{171}-2			R1F9		
		E/T:	100:1	25:1	6:1	100:1	25:1	6:1	100:1	25:1	6:1
1	BLCL + 3-28	–	0	0	0	15	8	3	89	78	35
	BLCL + 416-439	–	87	98	85	61	63	52	0	0	3
	BLCL unpulsed	–	1	0	0	10	7	2	0	0	0
	BLCL unpulsed	BLCL + 3-28	ND	ND	ND	ND	ND	ND	7	2	0
	BLCL unpulsed	BLCL + 416-439	16	5	3	18	11	2	ND	ND	ND
			R2F10								
		E/T:	100:1	25:1	6:1						
2	BLCL + 416-439	–	97	82	48						
	BLCL unpulsed	–	0	0	0						
	K562	BLCL + 416-439	0	5	0						
	K562	BLCL unpulsed	5	1	0						
			R1F9								
		E/T:	50:1	15:1	5:1						
3	BLCL + 3-12	–	80	39	8						
	BLCL unpulsed	–	0	4	2						
	R1F9 + 3-12	–	56	37	16						
	R1F9 unpulsed		0	0	0						
	BLCL unpulsed	BLCL + 3-12	1	3	1						
	BLCL unpulsed	R1F9 + 3-12	0	4	ND						
	R1F9 unpulsed	BLCL + 3-12	0	0	0						
	R1F9 unpulsed	R1F9 + 3-12	0	0	0						

Target cells were preincubated with or without peptide and used as ^{51}Cr labeled (hot) or unlabeled (cold) targets. Experiments were set up in triplicate wells to which 10^4 hot and if indicated 10^4 cold targets were added. Effector cells were then added at the indicated E/T (hot) ratios. ^{51}Cr release in the supernatants was determined after 4 h. Results are expressed as percent specific lysis.

cell killing. Effector cells were preincubated overnight with peptide and then added to peptide pulsed or nonpulsed target cells (Table VI). The preincubation of CTL did not result in lysis of unpulsed EBV-BLCL, suggesting that the formation of a functional TCR-peptide-MHC complex did not take place under these conditions. Lysis of peptide-pulsed target cells was not abrogated, showing that CTL peptide pretreatment did not block TCR-mediated recognition of MHC bound peptide. On the other hand, quantitative differences were observed: at higher E/T ratios, less efficient killing by pretreated CTL was seen, probably as a result of "cold effector" competition with peptide pulsed targets. At lower E/T ratios, killing was enhanced rather than inhibited. This could be accounted for by the preactivation of CTLs by peptide, and based on the above results, is not a consequence of enhanced TCR-antigen-MHC complex formation by TCR-bound free peptide.

Discussion

Until recently, cell-mediated immunity to intracellular organisms was thought to depend exclusively on CD4+ antigen-specific helper T lymphocytes: upon acti-

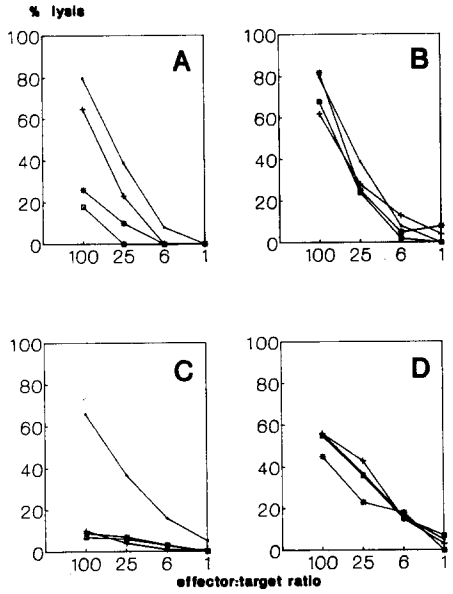

FIGURE 2. Inhibition of peptide-specific hot target cell lysis by peptide-pulsed or unpulsed cold target cells. Target cells were pulsed with 65-kD hsp peptide 3-12 (5 μg/ml) for 18 h. 5 × 10^3 ^{51}Cr-labeled target cells were mixed with unlabeled cold target cells at different cold/hot target ratios: (□) 50:1, (*) 15:1, (+) 5:1, (■) no cold target added. T cell clone R1F9 was used as effector cell in the experiment shown and added into wells at the indicated effector/hot target ratios. ^{51}Cr release in the culture supernatants was assessed after 4 h incubation. (*A*) Hot target:EBV-BLCL pulsed with peptide 3-12, Cold target:EBV-BLCL pulsed with peptide 3-12; (*B*) Hot target:EBV-BLCL pulsed with peptide 3-12, Cold target:EBV-BLCL not pulsed, (*C*) Hot target:R1F9 pulsed with peptide 3-12, Cold target:EBV-BLCL pulsed with peptide 3-12; (*D*) Hot target:R1F9 pulsed with peptide 3-12, Cold target:EBV-BLCL not pulsed.

vation by antigen/MHC class II, these cells would release lymphokines such as IFN-γ and thereby "help" or activate antimicrobial effector functions in the macrophage. This relatively simple scenario has recently been challenged, first by observations in a number of experimental infections in mice, including infections with *Mycobacteria sp.*, where CD4$^+$ and CD8$^+$ CTL could be isolated that were able to lyse macrophages (reviewed in reference 2). Those studies led us and others to reexamine the type of effector cells that were generated in the human immune response to mycobacteria. In summary, it was found that mycobacteria highly efficiently induced

TABLE VI

Effect of Peptide Preincubation of Effector Cells on Antigen-specific Lysis of Target Cells

			Effector: R1F9							
		Preincubated with:	No peptide				65-kD hsp peptide 3-28			
Target cell	Pulsed with 65-kD hsp peptide	E/T:	100:1	25:1	6:1	2:1	100:1	25:1	6:1	2:1
EBV-BLCL	3-28		100	96	43	14	54	49	64	44
EBV-BLCL	416-439		0	0	2	1	10	10	7	5
EBV-BLCL	–		9	8	0	0	0	0	2	0
			Effector: R2F10							
		Preincubated with:	No peptide				65-kD hsp peptide 416-439			
		E/T:	100:1	25:1	6:1	2:1	100:1	25:1	6:1	2:1
EBV-BLCL	3-28		0	3	0	1	9	4	0	0
EBV-BLCL	416-439		79	20	22	17	35	69	63	23
EBV-BLCL	–		0	4	7	2	9	0	0	6

Target cells or effector cells were preincubated with 5 μg/ml of the indicated antigens for 18 h and then washed three times. Assays were set up in triplicate (10^4 cells/well). The results are expressed as percent specific lysis.

antigen-specific, HLA-restricted CD4+ and CD8+ CTLs, as well as antigen nonspecific unrestricted killer cells (3–5). Each of these effector cells lysed macrophages. Recent studies in which rIL-2, rIFN-γ, or purified protein derivative (PPD) was injected into lepromatous leprosy patients' skin (29–31), and other studies in which in situ hybridization techniques were used (32) have also indirectly supported an important role for cytotoxic T cells in leprosy. Based on these new findings, the classical concept of cell-mediated immunity needs to be reconsidered, since it has become apparent that not only "macrophage-help" but also "macrophage-lysis" may be an effector mechanism in the cellular immune response to mycobacteria. We therefore wanted to gain further insight into the antigen specificity and function of CTL directed towards mycobacterial pathogens. We have demonstrated earlier that the mycobacterial 65-kD hsp is an important target antigen for CD4+ CTL (3, 5). These CTL were readily detectable after a single antigen exposure in vitro.

We have now analyzed the CTL response to this immunodominant antigen, using a panel of CTL clones. These CD4+ CD8− CTL recognize *M. leprae* 65-kD hsp peptides presented by HLA class II molecules and display strong cytotoxic activity towards specific peptide-pulsed EBV-BLCL target cells. Further experiments have extended these results to macrophages as target cells (data not shown). At least four different 65-kD hsp epitopes are able to trigger CTLs. Lysis of target cells was entirely specific and did not result in bystander lysis of EBV-BLCL, tumor cells or other CTL clones, although the CD4+ CTL release nonspecific serine esterases upon activation (unpublished observations). As far as we are aware, this is one of the first examples of peptide-specific CTL in a human infectious disease caused by intracellular parasites. Two other recombinant *M. leprae* antigens carry epitopes for CD4+ cytotoxic T cell clones, namely the recombinant 18-kD antigen and a newly defined molecule, 13B3 (33). These findings are in agreement with ours, and show that also non-65-kD antigens can be recognized by CD4+ CTL. We are presently also investigating other *M. leprae* antigens for their ability to serve as CTL target antigens. It will also be important to correlate our findings to the capacity of these CTL to produce lymphokines, since in mice (34) and maybe also in man (36) CD4+ Th1 but not Th2 cells possess lytic potential.

An unexpected and novel finding in the present study was that CD4+ CTLs are able to lyse CD4+ or CD8+ CTL clones that bear the proper HLA class II/peptide ligand. CTL are thought to be resistant to self-mediated lysis. Although during target cell killing CTL can avoid self-mediated killing by the unidirectional delivery of lytic granule compounds specifically to the target cell (36), also an active protection of CTL against lytic components has been proposed (e.g., 8–10), since CTL are unusually or sometimes even completely resistant to lysis by CTL or CTL products. For instance, cloned CTL are highly resistant to lysis by (other or self) CTL in a lectin-dependent cytotoxicity assay (14). In another study, a CTL clone efficiently lysed noncytotoxic helper T cells but only marginally lysed CTL targets, although the latter were properly recognized as evident from cold target competition experiments (12). A good correlation was found between cytotoxic capacity and resistance of CTL clones (13, 18). In contrast to their target cells, CTL themselves are highly resistant to lysis by purified CTL-derived cytotoxic granules (11, 14, 15), by highly purified perforin (17, 37). The same was found for primary, uncloned CTL (11).

It should be pointed out, however, that these studies were performed with murine

CTL that were almost always of the $CD8^+$ phenotype. These CTL may well differ from our human $CD4^+$ CTL. Our 65-kD hsp-specific CTL only lysed CTL targets that had been pulsed with free peptide but not with native hsp 65, suggesting that T cells do not endocytose and/or process native antigen. An exception may be antigens that specifically bind to recycling T cell membrane molecules. For instance, the gp120 HIV protein binds to CD4, is endocytosed, processed, and then presented to gp120-specific $CD4^+$ CTL that subsequently lyse $CD4^+$ class II^+ target cells (38).

Our CTL do not give bystander lysis. This finding contrasts with the recent suggestion that activated CTL have detectable affinity for self MHC, as determined by bystander lysis of autologous or syngeneic target cells (39). Differences in target and effector cells may explain these discrepant results. Peptide preincubation of effector cells did not lead to lysis of unpulsed targets, showing that if free peptide can bind to the TCR at all, it is unable to induce the formation of a functional peptide-MHC-TCR complex. Peptide preincubation of effector cells did not also abrogate lysis of peptide preincubated target cells, but led to more efficient lysis at lower E/T ratios, probably as a result of CTL preactivation by self presentation.

What is the possible in vivo relevance of CTL-mediated T cell killing? It is conceivable that CTL that lyse *M. leprae*-infected macrophages, become exposed to the intracellular breakdown products of the target cell, including polypeptides of the highly abundant 65-kD hsp of the infecting organism. These peptides might then bind to class II on the CTL surface and render them targets for CTL-mediated lysis. Mycobacterial granulomas in particular may contain abundant cellular and bacterial debris including such peptides. Cytolytic T-T interactions may be involved in downregulating local T cell responses and represent a novel immunoregulatory pathway that would exclusively be found in man, since murine T cells do not express MHC class II. Human T cell clones can indeed present antigen (our unpublished observations; 40), and cytotoxic T-T cell interactions may contribute to the depletion of $CD4^+$ T cells in HIV infections (38). Regulatory anticlonotypic cytotoxic T-T cell interactions have also been described in experimental autoimmune encephalomyelitis in mice (41).

Another immunoregulatory pathway in which mycobacterial antigen-reactive CTL may be involved is the downregulation of specific B cell responses (42). Because antigen-specific B cells express specific Ig receptors, they are much more efficient in the uptake, and hence presentation of processed antigen to T cells when compared with nonspecific B cells. It is conceivable that the CTL-mediated selective killing of specific B cells may, at least in part, explain why tuberculoid leprosy patients are good CTL responders to *M. leprae* and have low specific antibody titers, whereas the opposite is seen in lepromatous patients.

In conclusion, we have shown that mycobacterial peptide-specific HLA class II-restricted $CD4^+$ T cell clones display strong specific cytolytic activity towards both APCs, and, unexpectedly, $CD4^+$ and $CD8^+$ CTL clones, including themselves. Since, in contrast to murine T cells human T cells express class II, CTL-mediated T cell killing may represent a novel immunoregulatory pathway in man.

Summary

Mycobacterial antigens not only stimulate Th cells that produce macrophage-activating factors, but also $CD4^+$ and $CD8^+$ CTL that lyse human macrophages.

The mycobacterial recombinant 65-kD hsp was previously found to be an important target antigen for polyclonal $CD4^+$ CTL. Because of the major role of 65-kD hsp in the immune response to mycobacterial as well as autoantigens, we have studied CTL activity to this protein at the clonal level. HLA-DR or HLA-DQ restricted, $CD4^+CD8^-$ T cell clones that recognize different peptides of the *M. leprae* 65-kD hsp strongly lysed EBV-BLCL pulsed with specific but not irrelevant peptide. No bystander lysis of B cells, T cells, or tumor cells was seen. Target cell lysis could not be triggered by PMA + Ca^{2+} ionophore alone and depended on active metabolism. Interestingly, these $CD4^+$ CTL also strongly lysed themselves and other HLA-class II compatible $CD4^+$ (TCR-α/β or -γ/δ) or $CD8^+$ CTL clones in the presence of peptide, suggesting that CTL are not actively protected from CTL-mediated lysis. Cold target competition experiments suggested that EBV-BLCL targets were more efficiently recognized than $CD4^+$ CTL targets. These results demonstrate that hsp65 peptide-specific HLA class II-restricted $CD4^+$ T cell clones display strong peptide-dependent cytolytic activity towards both APCs, and, unexpectedly, $CD4^+$ and $CD8^+$ CTL clones, including themselves. Since, in contrast to murine T cells human T cells express class II, CTL-mediated T cell killing may represent a novel immunoregulatory pathway in man.

We thank Dave Anderson for generously making available peptides of the 65-kD hsp, Els Goulmy for providing $CD8^+$ CTL clones, Francesco Sinigaglia for providing T cell clone MGD5, Jan van Embden for providing the hsp 65, Dienne Bontrop-Elferink and Yolanthe Cornelisse for expert technical assistance, and René de Vries, Frits Koning, and Jim Kurnick for reviewing and Mariëtte Mulder for preparing the manuscript.

Received for publication 23 January 1990.

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