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Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4^{+} as well as CD8^{+} cytotoxic T lymphocytes in humans

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We investigated the cytotoxicity mechanisms of alloantigen-specific human CD4^{+} and CD8^{+} cytotoxic T lymphocytes (CTLs) using cells from family members with the Fas gene mutation. Alloantigen-specific CD4^{+} and CD8^{+} CTL bulk lines and clones were generated from 2 individuals by stimulation of their peripheral blood lymphocytes with allogeneic Fas$^{-/-}$ or Fas$^{+/-}$ cell lines that were established from B-lymphocytes of a patient with Fas deficiency and her mother, respectively. Both CD4^{+} and CD8^{+} CTL bulk lines and clones directed against allogeneic HLA antigens exerted cytotoxicity against Fas$^{-/-}$ and Fas$^{+/-}$ cells to almost the same degree. The cytotoxicity of CD4^{+} and CD8^{+} CTLs appeared to be Ca^{2+}-dependent and was completely inhibited by concanamycin A, an inhibitor of perforin-mediated cytotoxicity. Messenger RNAs for the major mediators of CTL cytotoxicity, Fas ligand, perforin, and granzyme B were all detected in these CD4^{+} CTLs with the use of the reverse transcriptase polymerase chain reaction. The majority of CD4^{+} CTL clones that showed Fas-independent cytotoxicity were T_H0, as determined by their cytokine production profile. These data, obtained with the use of a novel experimental system, clearly show that the main pathway of cytotoxicity mediated by alloantigen-specific human CD4^{+} as well as by CD8^{+} CTLs is granule exocytosis, and not the Fas/Fas ligand system. (Blood. 2000; 95:2352-2355)

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Introduction

There is no doubt that cytotoxic T lymphocytes (CTLs) directed against alloantigens play crucial roles in the development of graft rejection and graft-versus-host disease (GVHD) following organ transplantation. Therefore, understanding the cytotoxicity mechanisms of alloantigen-specific CTLs is essential in order to develop effective means of preventing rejection and GVHD.

Two main pathways in CTL-mediated cytotoxicity, ie, granule exocytosis mediated by perforin/granzymes and the Fas/Fas ligand system, have been identified.[1,2] The molecular mechanisms of CTL-mediated cytotoxicity have been studied extensively in murine systems with the use of various kinds of mutant and knockout mice, including Fas-mutant lpr,[3] Fas ligand-mutant gld,[4] perforin-deficient,[5-8] and granzyme-deficient[9] mice. These investigations have shown that CD8^{+} murine CTLs exert cytotoxicity mainly via granule exocytosis, whereas the main pathway of CD4^{+} murine CTL-mediated cytotoxicity is the Fas/Fas ligand system.[10,11] In contrast to these recent advances in our understanding of the mechanisms of murine CTL-mediated cytotoxicity, the details of human CTL-mediated cytotoxicity are still obscure owing to the lack of a suitable experimental system.

Recently, the presence of a disorder due to mutation of the *Fas* gene, the human counterpart of the lpr mouse, has been discovered.[12-14] Since the function of Fas is completely abrogated in these patients, this disease is expected to provide useful materials for investigating the functions of Fas in humans. In the present study, we examined the cytotoxicity mechanisms of alloantigen-specific human CD4^{+} and CD8^{+} CTLs using cells from family members with the *Fas* gene mutation. The results showed that in contrast to murine CD4^{+} CTLs, the major pathway of alloantigen-specific human CD4^{+} CTLs appeared to be granule exocytosis, and not the Fas/Fas ligand system, the same as that of CD8^{+} CTLs.

Materials and methods

Fas-deficient family members

Detailed information about the family members with the *Fas* gene mutation investigated in the present study has been reported previously.[15] A homozygous point mutation was present in the splicing acceptor site of intron 3 of the *Fas* gene of this patient (Fas$^{-/-}$). This mutation results in the skipping of exon 4 and complete loss of surface *Fas* expression. The patient's parents were both heterozygous for the same *Fas* gene mutation (Fas$^{+/-}$). The HLA types of each donor were as follows: Fas$^{-/-}$patient, A24/24, B48/48, Cw8/-, DRB1*0401/*0401; Fas$^{+/-}$ mother, A24/2, B48/35, Cw3/-, DRB1*0401/*08032; Fas$^{+/+}$ unrelated donor M.Y., A26/31, B61/62, Cw3/-, DRB1*0901/*1406; Fas$^{+/+}$ unrelated donor H.O., A24/24, B52/54, Cw1/-, DRB1*04051/*1502.

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Generation of alloantigen-specific CD4+ and CD8+ CTL bulk lines and clones

To generate alloantigen-specific CTL bulk lines and clones, peripheral blood mononuclear cells (PBMCs) from 2 unrelated individuals were cocultured with a mitomycin C (MMC)–treated Epstein-Barr virus–transformed B-lymphoblastoid cell line (B-LCL) established from the patient's PBMCs, or from the mother's PBMCs, in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated human AB-type serum, as described previously.[16] $CD4^+$ and $CD8^+$ T lymphocytes were isolated from PBMCs that had been stimulated with an allogeneic LCL for 6 days by means of magnetizable polystyrene beads coated with anti-CD4 and anti-CD8 monoclonal antibody (mAb) (DYNAL, Oslo, Norway), respectively. Uncloned $CD4^+$ and $CD8^+$ T lymphocytes were further cultured in RPMI 1640 medium supplemented with 10% human serum and interleukin (IL)–2, stimulated with the allogeneic LCL weekly 3 times, and then used as CTL bulk lines. Alloantigen-specific $CD4^+$ and $CD8^+$ CTL clones were established from PBMCs that had been stimulated with an allogeneic LCL by limiting dilution and had been continuously cultured in RPMI 1640 medium supplemented with 10% human serum and IL-2 with repeated stimulation by the allogeneic LCL, as described previously.[17]

Cytotoxic assays

Cytotoxicity was examined by standard 5-hour ^{51}Cr release assays using B-LCLs, which are sensitive to Fas-mediated cytotoxicity,[18,19] and freshly isolated sheep erythrocyte rosette-nonforming (E^-) cells, ie, B lymphocytes and monocytes, as target cells, as described previously.[20] In order to examine the Ca^{2+}-dependency of CTL-mediated cytotoxicity, ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) (Sigma, St Louis, MO) was added to the assay wells at 2 mmol/L with 2 mmol/L $MgCl_2$. In some experiments, concanamycin A (CMA) (Wako, Osaka, Japan) was added to assay wells at 10 nmol/L to inhibit perforin-mediated cytotoxicity.[21] Treatment of the cells with EGTA and CMA showed no toxic effect, as determined by the trypan blue exclusion test and ^{51}Cr release assay.

Detection of cytolytic mediator messenger RNA expression

Expression of the main mediators of cytolysis, including perforin, granzyme B, and Fas ligand, by T lymphocytes was examined by reverse transcriptase polymerase chain reaction (RT-PCR), as described previously.[22] Total RNA was extracted from CTLs that had been stimulated with allogeneic LCLs 5 days earlier, and complementary DNA (cDNA) was synthesized with Moloney murine leukemia virus reverse transcriptase. Amplification of the cDNAs by PCR was performed for 35 cycles with the use of the following primers: perforin, 5′-ACCAGCAATGTGCATGTGTC-TGTG-3′ and 5′-GAAGGAGGCCGTCATCTTGTGCTT-3′; granzyme B, 5′-TGCAGGAAGATCGAAAGTGCG-3′ and 5′-GAGGCATGCCATT-GTTTCGTC-3′; Fas ligand, 5′-ATAGGATCCATGTTTCTGCTCTTC-CACCTACAGAAGGA-3′ and 5′-ATAGAATTCTGACCAAGAGA-GAGCTCAGATACGTTGAC-3′. The expected lengths of the amplified cDNAs for the cytolytic mediators were 459 base pairs (bp), 180 bp, and 506 bp for perforin, granzyme B, and Fas ligand, respectively.

Cytokine production

For the assays of cytokine production, 1×10^6 clone cells and 3×10^5 MMC-treated allogeneic LCLs were suspended in 2 mL RPMI 1640 medium supplemented with 10% fetal calf serum and cultured in 16-mm wells. After 72 hours, the supernatants were collected from each well and were assayed for the production of various cytokines by enzyme-linked immunosorbent assay (ELISA) (Endogen, Woburn, MA).

Results

Cytotoxic activities of alloantigen-specific CD4+ and CD8+ CTL bulk lines and clones

We established 3 $CD4^+$ CTL bulk lines, 4 $CD4^+$ CTL clones, 3 $CD8^+$ CTL bulk lines, and 5 $CD8^+$ CTL clones from PBMCs of an unrelated individual, M.Y., which had been stimulated with the $Fas^{+/-}$ LCL, and 3 $CD4^+$ CTL bulk lines, 5 $CD4^+$ CTL clones, 3 $CD8^+$ CTL bulk lines, and 6 $CD8^+$ CTL clones from M.Y.'s PBMCs, which had been stimulated with the $Fas^{-/-}$ LCL. Similarly, 3 $CD4^+$ CTL bulk lines, 5 $CD4^+$ CTL clones, 3 $CD8^+$ CTL bulk lines, and 4 $CD8^+$ CTL clones were generated from PBMCs of an unrelated individual, H.O., which had been stimulated with the $Fas^{+/-}$ LCL, and 3 $CD4^+$ CTL bulk lines, 5 $CD4^+$ CTL clones, 3 $CD8^+$ CTL bulk lines, and 4 $CD8^+$ CTL clones were generated from H.O.'s PBMCs, which had been stimulated with the $Fas^{-/-}$ LCL. We used B-LCLs and E^- cells (B lymphocytes and monocytes) as the target cells, since they express both HLA class I and HLA class II molecules, which are targets of alloantigen-specific $CD8^+$ CTLs and $CD4^+$ CTLs, respectively. The same cytotoxicity patterns were detected in all bulk lines and clones within each group, and representative data for CTL bulk lines and clones from each group are presented in Table 1. Both $CD4^+$ and $CD8^+$ CTLs showed cytotoxicity against allogeneic LCLs, which were used as stimulators, but not against autologous LCL. Since the cytotoxic activity of $CD4^+$ CTL bulk lines and clones, and of $CD8^+$ CTL bulk lines and clones, were inhibited by anti–HLA class II and anti–HLA class I framework mAb, respectively (data not shown), the cytotoxicity mediated by these CTLs seemed to be directed against allogeneic HLA antigens. The degrees of cytotoxicity mediated by $CD4^+$ CTLs against $Fas^{+/-}$ and $Fas^{-/-}$ target cells were almost the same. Similarly, $CD8^+$ CTLs showed almost the same levels of cytotoxicity against $Fas^{+/-}$ and $Fas^{-/-}$ target cells. These data strongly suggest that the cytotoxicity mediated by human $CD4^+$ CTLs, as well as by $CD8^+$ CTLs, is Fas-independent.

Table 1. Cytotoxicity of alloantigen-specific $CD4^+$ and $CD8^+$ CTLs against allogeneic $Fas^{+/-}$ and $Fas^{-/-}$ cells and autologous cells

				% Specific Lysis of Target Cells*				
Donor	Stimulator	Phenotype	Effector	Allo ($Fas^{+/-}$) LCL	Allo ($Fas^{-/-}$) LCL	Allo ($Fas^{+/-}$) E−	Allo ($Fas^{-/-}$) E−	Auto LCL
M.Y.	$Fas^{+/-}$	CD4	Bulk #1-1	49.2	45.1	28.0	26.9	8.3
			Clone #1-1	68.3	64.9	43.7	48.1	2.7
			Clone #1-2	58.2	54.8	32.9	34.1	3.8
		CD8	Clone #2-1	66.3	62.0	43.7	41.0	2.9
	$Fas^{-/-}$	CD4	Bulk #3-1	34.9	31.8	21.8	25.3	9.5
			Clone #3-1	69.5	63.0	43.7	44.4	3.5
			Clone #3-2	53.2	57.6	34.5	32.0	3.4
		CD8	Clone #4-1	68.7	69.6	45.2	40.1	3.9
H.O.	$Fas^{+/-}$	CD4	Bulk #5-1	45.1	40.3	23.7	27.0	10.9
			Clone #5-1	56.6	53.0	32.1	35.5	4.4
			Clone #5-2	67.3	60.4	45.0	41.2	5.0
		CD8	Clone #6-1	54.9	51.9	39.9	34.1	3.5
	$Fas^{-/-}$	CD4	Bulk #7-1	56.2	52.9	29.6	31.2	11.4
			Clone #7-1	70.2	73.9	49.5	50.0	4.7
			Clone #7-2	62.7	68.9	34.1	38.0	6.3
		CD8	Clone #8-1	54.1	50.3	34.7	35.7	3.0

*Cytotoxicity of $CD4^+$ and $CD8^+$ CTL bulk lines and clones generated by stimulation of PBMCs isolated from unrelated donors with HLA class I and class II-mismatched $Fas^{+/-}$ or $Fas^{-/-}$ LCL against $Fas^{+/-}$ and $Fas^{-/-}$ target cells, as detailed in "Materials and methods." Effector/target ratios were 5:1.

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Expression of cytolytic mediators in alloantigen-specific CTL clones

RT-PCR revealed that the main cytolytic mediators of CTLs, including perforin, granzyme B, and Fas ligand, were all expressed in all of the $CD4^+$ and $CD8^+$ CTL bulk lines and clones examined. Representative RT-PCR data are shown in Figure 1. Expression of both perforin and Fas ligand was confirmed by flow cytometry using anti-perforin and anti-Fas ligand mAbs (data not shown).

Inhibition of cytotoxicity mediated by $CD4^+$ and $CD8^+$ alloantigen-specific CTL clones by EGTA and CMA

It is well known that perforin-mediated cytotoxicity is Ca^{2+}-dependent. As shown in Table 2, the cytotoxicity mediated by both $CD4^+$ and $CD8^+$ CTLs was inhibited completely by addition of the Ca^{2+}-chelating agent EGTA, indicating that their cytotoxicity is Ca^{2+}-dependent. In addition, the cytotoxicity mediated by both $CD4^+$ and $CD8^+$ CTLs appeared to be abrogated by the inhibitor of perforin-mediated cytotoxicity, CMA. These data show that the main pathways of cytotoxicity mediated by alloantigen-specific human $CD4^+$ and $CD8^+$ CTLs are both granule exocytosis.

Cytokine production by alloantigen-specific $CD4^+$ CTL clones

Alloantigen-specific $CD4^+$ CTL clones were cultured with or without $Fas^{+/-}$ and $Fas^{-/-}$ LCL, and the supernatants were analyzed for the production of IL-4, IL-10, and interferon (IFN)-γ. As shown in Table 3, the majority of $CD4^+$ CTL clones secreted all of these cytokines after stimulation with allogeneic LCL. Some of the $CD4^+$ CTL clones secreted IL-10 and IFN-γ, but not IL-4. Therefore, the majority of alloantigen-specific human $CD4^+$ CTLs generated in the present study can be classified as T_H0 type $CD4^+$ T-cell clones, with coexisting T_H1 clones.

Discussion

In contrast to the detailed data available on the cytolytic mechanisms of murine CTLs obtained previously with the use of various kinds of mutant and knockout mice, the mechanisms of cytotoxicity mediated by human CTLs are still obscure. Using a novel experimental system with cells from family members with the *Fas* gene mutation, which is considered to be the human counterpart of the lpr mouse mutation, we have clearly demonstrated that granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific human $CD4^+$ as well as $CD8^+$ CTLs.

Cytotoxicity mediated by alloantigen-specific human $CD4^+$ as well as $CD8^+$ CTLs are Ca^{2+}-dependent, since addition of the Ca^{2+}-chelating agent EGTA to the assay medium resulted in complete abrogation of cytotoxicity. Although Fas-dependent cytotoxicity has been reported to be Ca^{2+}-independent,[23] subsequent studies have revealed that even though the interaction between Fas ligand and Fas is Ca^{2+}-independent, T-cell receptor–dependent up-regulation of Fas ligand on the cell surface requires extracellular Ca^{2+}.[24] Therefore, it could not be concluded that cytotoxicity mediated by human $CD4^+$ CTLs is Fas-independent on the basis of the results obtained from the experiments using EGTA. However, we could conclude that cytotoxicity mediated by alloantigen-specific human $CD4^+$ CTLs is Fas-independent, since they lysed allogeneic $Fas^{-/-}$ and $Fas^{+/-}$ target cells, which shared an HLA haplotype, to the same degree. In addition, the cytotoxicity mediated by the $CD4^+$ CTLs was almost completely inhibited by treatment with a potent inhibitor of the perforin-based cytotoxic pathway, CMA. These data strongly suggest that the main pathway of alloantigen-specific human $CD4^+$ CTL-mediated cytotoxicity is granule exocytosis and that the cytolytic mechanism of human

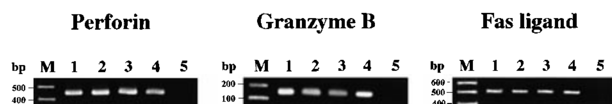

Figure 1. Expression of cytolytic mediators in alloantigen-specific human $CD4^+$ and $CD8^+$ CTLs. Expression of messenger RNAs (mRNAs) for perforin, granzyme B, and Fas ligand was investigated by RT-PCR. The mRNAs were extracted from $CD4^+$ clone #1-1 (lane 1), $CD4^+$ clone #3-1 (lane 2), $CD8^+$ clone #2-1 (lane 3), $CD8^+$ clone #4-1 (lane 4), and a negative control B-cell line (lane 5). Lane M shows 100-bp ladder markers.

Table 2. Inhibition of cytotoxicity mediated by alloantigen-specific CD4+ and CD8+ CTLs by EGTA and CMA

Donor	Stimulator	Phenotype	Effector	% Specific Lysis of Target Cells*					
				Allo LCL (Fas+/−)			Allo LCL (Fas−/−)		
				None	EGTA	CMA	None	EGTA	CMA
M.Y.	$Fas^{+/-}$	CD4	Bulk #1-1	45.8	0.8	1.5	43.0	0	0
			Clone #1-1	76.1	2.6	0.5	75.0	0.1	0.9
			Clone #1-2	52.8	0.3	0.1	49.8	0.1	0
		CD8	Clone #2-1	78.0	2.0	0.4	76.9	2.0	1.7
	$Fas^{-/-}$	CD4	Bulk #3-1	34.0	0	0.3	38.9	0.7	1.0
			Clone #3-1	69.2	0.1	0.5	65.8	0	0.8
			Clone #3-2	60.0	1.0	0	58.2	0	0
		CD8	Clone #4-1	78.9	2.1	1.0	76.3	0.8	0.3
H.O.	$Fas^{+/-}$	CD4	Bulk #5-1	42.9	0.5	1.6	40.5	0.6	0
			Clone #5-1	65.2	0.6	1.0	66.6	0	1.7
			Clone #5-2	54.2	0	0	51.0	0.4	0
		CD8	Clone #6-1	65.0	0.9	1.5	56.9	0.8	0
	$Fas^{-/-}$	CD4	Bulk #7-1	46.9	0.8	0	45.9	0.3	0
			Clone #7-1	67.7	2.0	1.6	69.8	1.1	1.3
			Clone #7-2	56.9	0.5	0.8	54.9	0.3	1.8
		CD8	Clone #8-1	75.2	2.9	0.7	70.8	0.8	0

*Cytotoxicity of $CD4^+$ and $CD8^+$ CTL bulk lines and clones generated by stimulation of PBMCs isolated from unrelated donors with HLA class I and class II-mismatched $Fas^{+/-}$ or $Fas^{-/-}$ LCL against $Fas^{+/-}$ and $Fas^{-/-}$ target cells in the presence or absence of EGTA or CMA, as detailed in "Materials and methods." Effector/target ratios were 5:1.

Table 3. Cytokine production by alloantigen-specific $CD4^+$ CTL clones

Donor	Effector	Stimulator	Cytokine production (pg/mL)*		
			IL-4	IL-10	IFN-γ
M.Y.	$CD4^+$ clone #1-1	Auto LCL	0	44	135
		Fas+/− LCL	118	3,492	1,812
	$CD4^+$ clone #1-2	Auto LCL	0	103	274
		Fas+/− LCL	123	3,684	1,952
	$CD4^+$ clone #3-1	Auto LCL	0	133	209
		Fas−/− LCL	0	3,812	1,924
	$CD4^+$ clone 3-2	Auto LCL	0	84	183
		Fas−/− LCL	156	3,060	2,224
H.O.	$CD4^+$ clone #5-1	Auto LCL	0	172	85
		Fas+/− LCL	118	3,260	2,048
	$CD4^+$ clone #5-2	Auto LCL	0	131	284
		Fas+/− LCL	123	3,668	2,096
	$CD4^+$ clone #7-1	Auto LCL	0	181	302
		Fas−/− LCL	0	3,700	2,068
	$CD4^+$ clone #7-2	Auto LCL	0	238	160
		Fas−/− LCL	157	3,760	1,696

*The $CD4^+$ clone cells were cultured with or without allogeneic LCL for 72 hours, and the concentration of each cytokine in the culture supernatant was determined by ELISA.

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$CD4^+$ CTLs generated in vitro is basically identical to that of $CD8^+$ CTLs.

The present data seem to be somewhat different from the recent findings obtained with murine systems indicating that granule exocytosis is the main pathway for $CD8^+$ CTL– and natural killer cell–mediated cytotoxicity, whereas most $CD4^+$ CTLs exert their cytotoxicity through the Fas/Fas ligand system.[10,11] In murine models of acute GVHD and graft rejection using granzyme B-deficient mice, it has been reported that granzyme B plays an important role in cytotoxicity mediated by $CD8^+$ CTLs, but not by $CD4^+$ CTLs.[25] The possibility that perforin-dependent cytotoxicity is restricted to some limited $CD4^+$ populations is unlikely, since all alloantigen-specific $CD4^+$ CTL bulk lines and clones examined showed Fas-independent cytotoxicity, and we have reported recently that herpes simplex virus–specific human $CD4^+$ CTLs also exert antigen-specific and HLA class II–restricted cytotoxicity through Fas-independent mechanisms.[16] In addition, we have recently found that cytotoxicity mediated by peptide-specific human $CD4^+$ CTL clones is also Fas-independent (unpublished data).

To clarify the subtype of $CD4^+$ CTL clones generated in the present study, we examined their cytokine production profile. It appeared that the vast majority of $CD4^+$ CTL clones generated were classified as T_H0-type clones, and some $CD4^+$ CTL clones secreted T_H1-type cytokines. Although the relationship between the cytokine production profile and cytolytic pathway of $CD4^+$ CTLs is unknown, the culture conditions of our experimental system might have favored the generation of T_H0 CTLs, which predominantly follow the granule exocytosis pathway. Therefore, further study is necessary to clarify the cytolytic pathway of human $CD4^+$ CTLs, which infiltrate various organs and exert cytotoxicity in vivo.

Although the mechanisms of graft rejection and GVHD are complex, CTLs directed against alloantigens undoubtedly play crucial roles in these serious problems associated with organ transplantation. Recent studies have shown that in addition to $CD8^+$ CTLs, $CD4^+$ CTLs are also important for induction of graft rejection and GVHD.[18,26,27] Thus, the present data indicate that we need to focus on the CTL granule exocytosis mechanism as well as the Fas/Fas ligand system when considering the development of novel methods for protection against alloreactivity.

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