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Biology of Cloned Cytotoxic T Lymphocytes Specific for Lymphocytic Choriomeningitis Virus: Clearance of Virus In Vivo†

JACQUELYN A. BYRNE* AND MICHAEL B. A. OLDSTONE
Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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Our data show that 1×10^7 to 1.5×10^7 lymphocytic choriomeningitis virus-specific, *H-2*-restricted cloned cytotoxic T lymphocytes (CTL) administered intravenously into acutely infected mice totally cleared virus from the spleens (10^4 to 10^5 PFU per spleen reduced to <50 PFU per spleen) by 24 h. This activity was genetically restricted in that cloned CTL could reduce titers of infectious virus in syngeneic C57BL/6 mice but not allogeneic BALB/c mice. Dose-response analysis indicated that at least 3×10^6 to 5×10^6 cloned CTL injected intravenously were needed to reduce significant amounts of infectious virus in the spleens. No infectious virus could be recovered from the spleens for at least 4 days after injection of cloned CTL. Hence, CTL play a major role in elimination of infectious virus from spleens during lymphocytic choriomeningitis virus infection. Our results also indicate that cloned CTL propagated in vitro for long periods of time can mediate a biologically relevant effect in vivo. These cells should be of considerable value in defining the precise manner in which CTL bring about control of viral infection, analyzing lymphocyte trafficking, and the potential use of cloned CTL in immunotherapy against viral disease.

Lymphocytic choriomeningitis virus (LCMV) naturally infects and replicates in the murine host. Work from a number of laboratories has demonstrated that infection of adult mice with LCMV by intraperitoneal (i.p.) or intravenous (i.v.) inoculation usually leads to the generation of a protective immune response and clearance of infectious virus (4, 5). In contrast, a similar dose of virus inoculated intracerebrally causes an immune-mediated choriomeningitis and death (14). This viral infection provides an excellent model to study how the interaction of a virus with the host immune system can lead to a protective immune response and clearance of viral infection or, alternatively, to virus-induced immune response disease (1, 2, 4, 5, 7, 18, 19, 27). Experiments with LCMV have provided important insights toward understanding cell-mediated immune responses generated against viral infection (27) and the role that these activities play both during recovery from infection and during mediation of pathological processes (6, 9, 11, 24).

Upon infection of immunocompetent adult animals, a potent cytotoxic T lymphocyte (CTL) response is generated (7, 16). By using LCMV as a model, it was first demonstrated that the CTL response is virus specific (16) as well as *H-2* restricted (25, 26) in its activity. In later studies, the importance of a T cell-mediated response in the clearance of infectious virus (17) and in the induction of immune-mediated disease has been demonstrated (6, 11, 24). Mims and Blanden (17) and Zinkernagel and Welsh (27) have shown that spleen cells taken from adult mice 7 to 8 days after they were inoculated with LCMV and adoptively transferred to acutely infected mice cleared virus from the infected mice. The same activated spleen cell population has been demonstrated to transfer the viral-induced immune response disease when injected into immunocompetent adult mice that were inoculated intracerebrally with LCMV (11). In both cases, a necessary cell population in the preparation was found to be the T lymphocytes, which were only effective when donors and recipients shared *H-2*K or *H-2*D region specificities (6, 9, 27). However, because heterogeneous populations of primed T cells rather than homogeneous cloned populations were used, the exact nature of LCMV-specific T cell(s) or other lymphocytes involved directly or indirectly in these processes could not be clearly established. The development of techniques for in vitro cloning and long-term culture of T lymphocytes now allows us to obtain large numbers of homogeneous cloned T cells with specific functions (12, 13, 23). These homogeneous populations can be used to ask questions concerning the specific contributions of various T lymphocyte subsets to the control of viral infection or, alternatively, the induction of pathological processes. The information obtained would add to our understanding of the pathogenesis of viral infections as well as aid us in devising appropriate immunization protocols to protect against viral infection. Such CTL clones could also be of value as unique tools for defining CTL trafficking and target homing as well as probes to uncover the molecular basis of CTL-mediated activity in vivo.

Here we report the first demonstration that LCMV-specific, *H-2*-restricted CTL clones can act in vivo and demonstrate their ability to clear infectious virus from acutely infected mice.

MATERIALS AND METHODS

Virus stock and assay for virus PFU. The Armstrong CA 1371 strain of LCMV (LCMV Arm) was used in all experiments. Virus was plaque purified on Vero cells, virus stocks were grown in BHK cells, and titers of virus stocks were determined on Vero cell monolayers as previously described (3). Spleens whose titers were to be determined for infectious virus were removed asceptically and frozen in Eagle minimal essential medium supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 30 min), 1 mM glutamine, penicillin, streptomycin, and amphotericin B. To assay for virus titers, samples were thawed, homogenized,

* Corresponding author.
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and centrifuged (1,000 rpm for 10 min at 4°C) to remove all debris, and the titers of the supernatants were determined on Vero cell monolayers as previously described (3).

Mice. Male C57BL/6 and BALB/c WEHI (BALB/c) mice were obtained from the breeding colony at Scripps Clinic and Research Foundation and used when they were 5 to 8 weeks of age.

Generation and cloning of CTLs. Generation and cloning of LCMV-specific, *H-2*-restricted CTLs have been described previously (4a). Viable spleen cells (8×10^6) from mice injected 4 weeks previously with 10^5 PFU of LCMV Arm i.p. were cultured with 2×10^5 LCMV Arm-infected syngeneic peritoneal exudate cells in 2 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM glutamine, penicillin, streptomycin, and 5×10^{-5} M β-mercaptoethanol per 16-mm well (Costar plates). On day 9, responder cells were harvested and cloned by a limiting dilution technique: graded numbers of responder cells (0.1 to 100 per well) were cultured in 96-well plates (Costar) containing 10^6 LCMV Arm-infected syngeneic spleen cells per well (irradiated with 2,000 rads from a ^{137}Cs source) in RPMI-S medium containing 30% concanavalin A supernatant; 5×10^6 viable mouse spleen cells per ml were incubated for 24 h at 37°C in the presence of 3 μg of concanavalin A supernatant per ml supplemented with 10 mg of α-methyl-D-mannoside per ml. Cultures were fed on days 5, 8, and 11. On day 14, wells were scored visually for growing cells. Responder cell cultures diluted so that less than 20% of the wells were positive for growth were expanded to 16-mm wells containing 10^5 irradiated (2,000 rads from a ^{137}Cs source) syngeneic macrophages infected with LCMV Arm (multiplicity of infection, 2 PFU per cell) and 4×10^6 syngeneic irradiated spleen cells per well in 2 ml of RPMI-S medium supplemented with 30% concanavalin A supernatant. Clones were maintained by weekly passage to replicate 16-mm wells. Cytotoxicity assays and adoptive transfer experiments were done with cells harvested 5 to 7 days after passage. Clone 11-5 was maintained in an in vitro culture for over 1 year. Precise details of cloning maintenance and phenotyping are given elsewhere (4a).

In vitro cell-mediated cytotoxicity. For cytotoxicity testing, targets were as follows: MC57 cells infected with LCMV Arm or Pichinde virus (48 h; multiplicity of infection, 0.5 PFU per cell), BALB/c CL-7 cells infected with LCMV Arm (24 h; multiplicity of infection, 5 PFU per cell), and uninfected MC57 and BALB/c CL-7 cells. Target cells were labeled by incubating 2×10^6 cells with 200 μl of sodium [^{51}Cr]chromate for 1 h at 37°C. Washed (three times) target cells (2×10^4) were mixed with washed (two times) effector cells at the designated effector-to-target ratio in 0.2 ml of RPMI-S supplemented with 10% heat-inactivated fetal bovine serum, 1 mM glutamine, penicillin, and streptomycin in 96-well flat-bottom plates. In place of effectors, 100 μl of RPMI-S medium was used to measure spontaneous lysis and 100 μl of 1% Nonidet P-40 was used to measure total lysis. Plates were incubated for 6 h at 37°C and centrifuged at 500 rpm at 4°C for 5 min, after which 0.1-ml fractions of the supernatant were collected and counted for radioactivity. The percentage of specific ^{51}Cr released was calculated by:

$$100 \times \frac{(\text{cpm with effectors}) - (\text{cpm of spontaneous lysis})}{(\text{cpm of total lysis}) - (\text{cpm of spontaneous lysis})}$$

where cpm is counts per minute.

In vivo assay for CTL clone activity. Recipient mice were injected on day 0 with 2×10^3 PFU of LCMV Arm i.v. and adoptively transferred i.v. with cells on day 1. On days 2, 4, and 5, mice were sacrificed, and the spleens were assayed for titers as described above. Where designated, recipient mice received 800 rads from a ^{137}Cs source on day 0 immediately before virus injection.

RESULTS

In vitro cytotoxic activity of CTL clones. Fifteen T lymphocyte clones were derived from C57BL/6 mice as described above and tested for their ability to kill a panel of target cells in a ^{51}Cr-release assay (4a). Of 14 clones, 13 were found to be cytotoxic for the appropriate LCMV-infected syngeneic target cells. One CTL clone (11-5) was selected for use in in vivo experiments due to its rapid in vitro growth and high level of specific cytotoxic activity. Clone 11-5 was highly cytotoxic for LCMV-infected MC57- (H-2^b) infected targets (Table 1). Its cytotoxic activity in vitro was LCMV specific (no killing of syngeneic targets that were either uninfected or infected with another arenavirus, Pichinde virus) and H-2^b restricted (no killing of LCMV-infected allogeneic, H-2^d target cells). The failure of clone 11-5 to kill various target cells was not a result of the target cells not being susceptible to lysis since they could all be killed by the appropriate primed spleen cell population. In addition, clone 11-5 failed to kill the LCMV-infected *H-2* recombinant target, B10.A(5R) (H-$2K^bD^d$), indicating restriction to the *H-2*D end of the major histocompatibility complex (4a). Analysis by fluorescence-activated cell sorting demonstrated that the surface phenotype of clone 11-5 is Thy-1.2$^+$, Lyt-1.2$^-$, Lyt-2.2$^+$.

TABLE 1. Cytotoxic activity of CTL clone 11-5

Effector	E:T[a]	% Specific ^{51}Cr release from target cells: MC57 (H-$2K^bD^b$) LCMV Arm	MC57 Pichinde	MC57 Uninfected	B10.A(5R) (H-$2K^bD^d$) LCMV Arm	B10.A(5R) Uninfected	BALB/c CL-7 (H-$2K^dD^d$) LCMV Arm	BALB/c CL-7 Uninfected
Clone 11-5	2.5:1	93	2	3	0	5	0	0
Clone 11-5	0.5:1	73	0	0	0	6	0	0
BALB/c (LCMV)[b]	50:1	NT[c]	NT	NT	NT	NT	64	3
BALB/c (uninfected)[d]	50:1	NT	NT	NT	NT	NT	0	0
C57BL/6 (Pichinde)[e]	50:1	3	47	3	NT	NT	NT	NT
C57BL/6 (uninfected)	50:1	1	1	1	NT	NT	NT	NT

[a] E:T, Effector-to-target cell ratio.

[b] Spleen cells from BALB/c mice injected i.p. 7 days previously with 10^5 PFU of LCMV.

[c] NT, Not tested.

[d] Spleen cells from uninfected mice.

[e] Spleen cells from C57BL/6 mice injected i.p. 8 days previously with 10^5 PFU of Pichinde virus.

Activity of cloned CTL in vivo. We assessed the ability of cloned CTLs to clear virus during acute infection by using an adoptive transfer model, as previously described by others (17, 27), to study the effects of primed spleen cells on acute LCMV infection. C57BL/6 mice (5 to 8 weeks old), injected i.v. with LCMV Arm and sacrificed 48 h later, had approximately 10^4 to 10^5 PFU of virus in their spleens (Table 2). However, these virus titers were significantly reduced by adoptive transfer of 10^8 spleen cells from mice injected 7 days previously with LCMV (7-day immune spleen cells) but not by adoptive transfer of an equal number of normal spleen cells, which confirms previous reports (27). Adoptive transfer of 8×10^6 CTLs of clone 11-5 reduced the titers of infectious virus in the spleens of recipient mice by at least 99% (Table 2), thus demonstrating that cloned T lymphocytes with cytotoxic activity in vitro mediate a biologically relevant effect in vivo in acutely infected animals.

To test whether transfer of cloned CTLs could maintain the clearance of virus for a period of time, mice were adoptively transferred with cloned CTLs 24 h after infection with LCMV and sacrificed at 2, 4, and 5 days postinfection (1, 3, and 4 days, respectively, after adoptive transfer of cloned CTLs) (Table 3). Immediately before infection, recipient mice received 800 rads from a ^{137}Cs source to prevent contributions from the host immune response. Mice receiving 1.5×10^7 cloned CTLs had no detectable infectious virus in their spleens at 2, 4, or 5 days after LCMV infection. In contrast, mice infected with LCMV and not receiving cloned CTLs had high titers of virus in their spleens during the same time period. Thus, adoptively transferred cloned CTLs were able to maintain the clearance of virus over at least a several-day time span. When the number of CTLs needed to clear virus was assessed, we found that adoptive transfer of 1×10^7 CTLs reduced the titer >99%, 3×10^6 CTLs reduced the titer 50 to 90%, and 1×10^6 CTLs had a minimal effect (data not shown). However, ongoing experiments in which cloned CTLs labeled with ^{51}Cr are transferred into acutely infected mice indicate that only 2 to 5% of such cells are present in the spleen between 2 and 16 h after injection, suggesting that only a fraction of the cells injected migrate to the spleen and totally clear virus from that organ (J. A. Byrne and M. B. A. Oldstone, manuscript in preparation).

TABLE 2. Reduction of virus titers in the spleens of acutely infected animals by adoptive transfer of cloned CTLs[a]

Expt no.	Cells transferred	Dose per mouse	Log_{10} PFU per recipient spleen[b]
1	Nil	Nil	3.9
			4.1
			4.1
			4.3
	C57BL/6 7-day immune spleen[c]	1×10^8	<1.7
			<1.7
			<1.7
			<1.7
	C57BL/6 normal spleen[d]	1×10^8	5.0
			4.3
			4.6
	Clone 11-5 (*H-2*^{b})	8×10^6	<1.7
			<1.7
			<1.7
		5×10^6	<1.7
2	Nil	Nil	4.1
			3.8
			4.0
	C57BL/6 7-day immune spleen	1×10^8	<1.7
			<1.7
			<1.7
	Clone 11-5	8×10^6	<1.7
			2.0
			<1.7

[a] C57BL/6 recipient mice were injected on day 0 with 2×10^3 PFU of LCMV-Arm i.v. and adoptively transferred with cells i.v. on day 1. On day 2, mice were sacrificed, and spleens were assayed for virus titers by plaque assay on Vero cells.

[b] Individual spleens were homogenized, and virus titers were determined by plaque assay on Vero cells. Each number represents data from a single mouse.

[c] Donor mice were injected with 10^5 PFU of LCMV Arm i.v. 7 days before sacrifice and adoptive transfer.

TABLE 3. Maintenance of reduction of virus titers in the spleens of acutely infected animals by adoptive transfer of cloned CTLs[a]

Cells transferred	Dose per mouse	Log_{10} PFU per recipient spleen on the following days, postinfection[b]: 2	4	5
Nil	Nil	6.0	5.4	4.3
		5.6	5.0	4.5
		5.6	5.4	4.3
		5.5	5.4	4.3
Clone 11-5	1.5×10^7	<1.7	<1.7	<1.7
		<1.7	<1.7	<1.7
		<1.7	<1.7	<1.7
				<1.7
C57BL/6 7-day immune spleen[c]	1×10^8	NT[d]	<1.7	NT
			<1.7	
			<1.7	
			<1.7	

[a] C57BL/6 recipient mice were given 800 rads from a ^{137}Cs source and injected with 2×10^3 PFU of LCMV Arm i.v. on day 0 and adoptively transferred with cells i.v. on day 1. On days 2, 4, and 5, mice were sacrificed, and spleens were assayed for virus titers by plaque assay on Vero cells.

[b] Individual spleens were homogenized, and virus titers were determined by plaque assay on Vero cells. Each number represents data from a single mouse.

[c] C57BL/6 donor mice were infected with 10^5 PFU of LCMV Arm i.p. 7 days before sacrifice and adoptive transfer.

[d] NT, Not tested.

***H-2* restriction of CTL clones in vivo.** Since the cytotoxic activity of CTL clones is genetically restricted in vitro (no killing of BALB/c CL-7, *H-2*^{d} cells infected with LCMV [Table 1]), we next tested the genetic restriction of the activity of this clone in vivo in two experiments (Table 4). As in the previous experiments, injection of C57BL/6 mice with clone 11-5 or 7-day immune spleen cells resulted in significant reductions of virus titers. In contrast, clone 11-5 did not lower virus titers in the spleens of acutely infected allogeneic BALB/c mice. Thus, clone 11-5 is only capable of eliminating infectious virus when functioning in the appropriate genetic environment, presumably involving recognition of viral antigen in the context of the proper *H-2*^{b} gene products. The fact that syngeneic immune spleen cells injected into BALB/c mice decreased titers of infectious virus demonstrates that BALB/c LCMV-immune spleen cells also contain a population of cells capable of eliminating infectious virus from the BALB/c recipient within the same time period. The inability of clone 11-5 to reduce virus titers in the allogeneic recipient was not due to the fact that LCMV-immune cells were unable to function similarly in infected BALB/c mice.

TABLE 4. Genetic restriction of adoptively transferred cloned CTLs reducing virus titers in animals acutely infected with LCMV

Expt no.	Recipient[a]	Cells transferred	Dose per mouse	Log_{10} PFU per recipient spleen[b]
1	C57BL/6 (*H-2*^{b})	Nil	Nil	3.4
				4.4
				4.1
	C57BL/6	C57BL/6 7-day immune spleen[c]	1×10^{8}	<1.7
				<1.7
				<1.7
	C57BL/6	Clone 11-5 (*H-2*^{b})	1×10^{7}	<1.7
				2.0
	BALB/c (*H-2*^{d})	Nil	Nil	4.8
				4.6
				4.6
	BALB/c	BALB/c 7-day immune spleen	1×10^{8}	<1.7
				<1.7
				<1.7
	BALB/c	Clone 11-5	1×10^{7}	4.3
				4.6
				4.6
2	C57BL/6	Nil	Nil	4.3
				4.3
				5.3
	C57BL/6	Clone 11-5	1×10^{7}	2.1
				2.2
				2.6
	BALB/c	Nil	Nil	5.3
				5.6
				5.4
	BALB/c	BALB/c 7-day immune spleen	1×10^{8}	<1.7
				<1.7
	BALB/c	Clone 11-5	1×10^{7}	5.3
				5.3
				5.0

[a] Treatment of recipient mice was identical to that as described in footnote *a* of Table 2.
[b] Individual spleens were homogenized, and virus titers were determined by plaque assay on Vero cells.
[c] Donor mice were injected with 10^{5} PFU of LCMV Arm i.p. 7 days before sacrifice and adoptive transfer.

DISCUSSION

Our adoptive transfer experiments with a cloned CTL line demonstrate that T lymphocytes with virus-specific, *H-2*-restricted cytotoxic activity in vitro can clear and maintain clearance of virus infection for at least 4 days after injection. Furthermore, the in vivo activity is genetically restricted; cloned CTLs clear virus from syngeneic C57BL/6 mice but are unable to clear virus from allogeneic recipients.

Considerable information has been reported concerning the requirements for generation of the response of CTLs during viral infection as well as the specificity of that response. However, analysis of how the activated CTLs might contribute to the elimination of infectious virus in the infected host has been difficult without the availability of pure populations of CTLs. Previous studies on LCMV infection by adoptive transfer of heterogeneous spleen cell populations have demonstrated a necessary cell population for viral clearance to be T lymphocytes whose activity was restricted to the K or the D end of the major histocompatibility complex (27). From such experiments, it was not clear whether one or more T cell populations (or another cell population in addition to the T lymphocyte) was necessary. The data presented here indicate that the CTL is sufficient for clearance of acute LCMV infection and that the cooperation of several populations of primed T lymphocytes mediating different immunological functions is unnecessary. Furthermore, a significant drop in virus titer was accomplished through the activity of a single CTL clone which presumably recognized one viral determinant in the context of a single major histocompatability complex epitope. Thus, a heterogeneous mixture of CTL clones with various specificities for virus plus *H-2* gene products is not required for clearance of virus.

Our data extend the observations of Lin and Askonas (15), who reported that administration of cloned influenza-specific CTLs was capable of reducing virus titers in the lungs of infected animals and prolonging their survival, and of Sethi et al. (20), who found that cloned CTLs could protect mice against a lethal challenge of herpes simplex virus type 1. In contrast to those models, LCMV naturally infects and replicates in mice, so we have demonstrated the function of cloned CTLs against a viral infection in its natural host. Furthermore, we have provided the first evidence that the reduction of virus titers in vivo by a cloned CTL is genetically restricted.

Clone 11-5 was found by fluorescent-activated cell sorter analysis to be Thy-1^{+}, Lyt-1.2^{-}, Lyt-2.2^{+}. Varho et al. (22) have found the cytotoxic Lyt-1^{-}23^{+} spleen cell population to be ineffective in eliminating infectious virus from LCMV-infected mice and concluded that the Lyt-1^{+}23^{+} population may be active in this respect. Although we cannot exlude the possibility that the Lyt-1^{+}23^{+} population (which may also be cytotoxic) may be capable of mediating virus reduction in vivo, our results clearly demonstrate that a cell which is cytotoxic in vitro and is Lyt-1^{-}2^{+} can eliminate infectious virus in vivo.

There have been recent reports that cloned CTLs which have been maintained in in vitro culture for long periods of time have alterations in their trafficking to the appropriate lymphoid organs and may lack appropriate cell surface receptors that are important for proper homing (8, 10, 21). Preliminary studies with clone 11-5 have indicated that only a small percentage (2 to 5%) of the cells can be found in the spleen at any given time. Nevertheless, we found that the small percentage of cells that circulate to the spleen are capable of mediating and maintaining a significant reduction in virus titers. Detailed study of the trafficking patterns of such cloned CTLs, coupled with analysis of their ability to influence virus replication in other tissues, should allow us to assess how any alteration in trafficking would effect the ability of cloned CTLs to function properly in the control of virus infection. This would provide valuable information on the relation of trafficking patterns to normal lymphocyte functions as well as the potential use of cloned cells for adoptive immunotherapy.

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