

IN VIVO EFFECTOR FUNCTION OF INFLUENZA VIRUS-
SPECIFIC CYTOTOXIC T LYMPHOCYTE CLONES IS
HIGHLY SPECIFIC

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A role for cytotoxic T lymphocytes (CTL)¹ in the host defense to viral infection has been postulated based on their capacity to specifically lyse virus-infected syngeneic cells in culture. However, direct evidence for an *in vivo* effector function of this T lymphocyte subset is limited. Among the most definitive demonstrations of CTL activity *in vivo* are the models of host defense to primary virus infection in mice (1-4). In the murine influenza system, several groups (5-7) have shown that adoptive transfer of immune cells possessing cytotoxic activity to influenza virus-infected mice promotes their recovery and reduces pulmonary virus titers. The cells with *in vivo* effector activity bore the Thy-1 and Lyt-2 surface markers, required K/D but not I region homology between donor and recipient for *in vivo* activity, and exhibited significant influenza virus-specific cytotoxic activity *in vitro* (7). Based on these criteria, it was deduced that CTL represented the T cell subclass responsible for conferring protection in this viral system. However, since heterogeneous donor cell populations were used in these studies, neither the precise identity of the effector cell nor its mechanism of action *in vivo* could be ascertained. Lin and Askonas (8) then showed that an influenza virus-specific cloned CTL line, which depended solely on T cell growth factor (TCGF) for *in vitro* propagation, reduced lung virus titers and prolonged survival after adoptive transfer to influenza virus-infected mice. However, in a recent study (9), another cloned influenza-specific CTL line examined by this group failed to alter the course of influenza infection upon adoptive *in vivo* transfer.

Recently, there have been a series of reports (10-12) documenting the secretion of lymphokines, most notably interferon- γ , by CTL populations. These findings support the hypothesis of Zinkernagel and Althage (13) that, in addition

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¹*Abbreviations used in this paper:* BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; DMEM, Dulbecco's modified minimal essential medium; FBS, fetal bovine serum; HA, influenza hemagglutinin protein; [³H]TdR, tritiated thymidine; IL-2, interleukin 2; 2-ME, 2-mercaptoethanol; MEM, minimal essential medium; NBS, newborn bovine serum; PBS, phosphate-buffered saline; PFU, plaque-forming units; TCGF, T cell growth factor.

to direct cytolysis, virus-specific CTL could exert their antiviral effect *in vivo* by release of lymphokines with direct or indirect antiviral activity. Since secretion of lymphokines by the CTL is triggered by major histocompatibility complex-restricted recognition of specific antigen (e.g., virus-infected cells), these lymphokines might serve as a primary antiviral effector mechanism *in vivo*. Thus, the relative importance of cytolysis and soluble factors released by CTL as the CTL-specific antiviral effector mechanism is not yet defined.

Our laboratory has isolated and characterized murine CTL clones directed to type A influenza virus (14). In this report, we examine the *in vivo* antiviral effector activity of cloned CTL adoptively transferred into syngeneic mice lethally infected with type A influenza virus. We show that two influenza virus-specific CTL clones do protect recipient mice from death and mediate recovery from primary viral pneumonia. One CTL clone, which recognizes only type A influenza viruses of the H2N2 subtype *in vitro*, selectively promotes recovery from lethal influenza infection and reduces pulmonary virus titers in a subtype-specific fashion. In contrast, the other CTL clone that exhibits cross-reactive recognition of type A influenza virus independent of subtype *in vitro*, likewise protects mice infected by either of two different virus subtypes. When mice are simultaneously infected with lethal doses of influenza virus strains of two different subtypes, the subtype-specific CTL clone cannot promote recovery. Furthermore, in these dual-infected animals this CTL clone can only reduce the pulmonary virus titer of the strain representative of the subtype recognized by this CTL clone. The implications of these findings to the *in vivo* effector mechanism of CTL are discussed.

Materials and Methods

Animals. Male BALB/cByJ (H-2^d) mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and used at 7 wk of age as recipients in adoptive transfer experiments.

Viruses. Influenza virus strains A/JAP/57 [A/Japan/305/57 (H2N2)] and A/MEL/35 [A/Melbourne/35 (H1N1)] were grown in the allantoic cavity of 10-d-old embryonated chicken eggs and stored as infectious allantoic fluid as previously described (15).

Cell Lines. The P815 (H-2^d) and MDCK cell lines were maintained in culture in Dulbecco's modified minimal essential medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with antibiotics and 10% (vol/vol) fetal bovine serum (FBS) or 10% (vol/vol) newborn bovine serum (NBS), respectively. The MDCK cell line was kindly provided by Dr. Virginia S. Hinshaw of the St. Jude Children's Research Hospital, Memphis, TN.

Neutralizing Antisera. Rabbit anti-A/MEL/35 virus antiserum was generously provided by Dr. Robert Webster of the St. Jude Children's Research Hospital. Rabbit antiserum to purified A/JAP/57 virus hemagglutinin (HA) was prepared as described previously (16). Both antisera had neutralizing titers for their respective viruses of >1:100. A 1:10 dilution of each antiserum was prepared in phosphate-buffered saline (PBS). A 1 h incubation at 4°C of rabbit anti-A/MEL antiserum with infectious A/MEL/35 or A/JAP/57 virus completely neutralized infectious A/MEL and did not affect A/JAP virus titer, as assayed by plaque formation on MDCK cell monolayers (see below). Antiserum directed to the A/JAP/57 HA, under the same conditions, completely neutralized A/JAP virus without affecting the A/MEL virus titer.

Cloned CTL Lines. The procedures developed to establish and maintain influenza virus-specific cloned CTL lines are described in detail elsewhere (14). Briefly, spleen cells from BALB/c mice immunized intravenously with infectious A/JAP/57 virus 3 wk earlier were stimulated *in vitro* with A/JAP/57 virus-infected, γ -irradiated (2,000 rad) BALB/c

spleen cells. Cloned lines were isolated by limiting dilution of viable cells from tertiary cultures. The clones were selected, expanded, and maintained in the presence of A/JAP/57-infected, irradiated BALB/c spleen cells in Iscove's modified Dulbecco's medium (Gibco Laboratories) supplemented with 10% (vol/vol) FBS (Sterile Systems, Inc., Logan, UT), 5×10^{-5} M 2-mercaptoethanol (2-ME), antibiotics, and 10% (vol/vol) crude rat T cell growth factor (TCGF). The lines were serially passaged every 5–6 d in six-well cluster tissue culture plates (No. 3506; Costar, Cambridge, MA) containing 20×10^6 infected, irradiated BALB/c spleen cells and 10% TCGF.

Intranasal Virus Inoculation of Mice. Mice, under light anesthesia from a diethyl ether/chloroform (2:1) mixture, were inoculated by placing 50 μ l of virus suspension diluted in cold PBS to the nose pad using a 50 μ l Eppendorf micropipette. Each mouse received a dose of 10 LD₅₀ of virus. In the dual-infection experiment described below (Results), each mouse was inoculated with 50 μ l of a mixture containing 10 LD₅₀ A/JAP/57 and 10 LD₅₀ A/MEL/35 viruses. LD₅₀ values were calculated according to a modified Spearman and Karber method (17).

Adoptive Transfer Procedure. Viable CTL cloned cells, expanded in six-well cluster tissue culture plates as described above for routine passaging, were separated from dead cells according to the method of Davidson and Parish (18). The cells were washed twice with Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) FBS, washed once with MEM, and resuspended in MEM at a concentration of 2×10^7 cells/ml. A 0.5 ml vol of this resuspension (i.e., 10^7 cells) was injected intravenously via the tail vein into each mouse. Virus-infected control mice each received 0.5 ml MEM i.v. Adoptive transfers of CTL cloned cells were carried out within 1 h after intranasal inoculation of virus.

Lung Virus Titration by MDCK Cell Plaque Assay. Lungs were aseptically removed at the bronchi, snap-frozen in liquid nitrogen, and stored at -70°C until ready for titration. The lungs were thawed at 4°C in 2 ml PBS supplemented with antibiotics and 0.75% bovine serum albumin (BSA) (670–5260; Gibco Laboratories). Lungs were then manually homogenized (Dounce tissue grinder) and centrifuged (3,000 g for 15 min, 4°C). 10-fold dilutions of lung extract supernatants were prepared in PBS supplemented with 0.75% BSA, 0.1 g/l MgCl₂, 0.1 g/l CaCl₂, and antibiotics. MDCK cell monolayers were prepared by seeding 2×10^5 MDCK cells in 2 ml DMEM supplemented with 10% NBS into individual wells of 12-well cluster tissue culture plates (No. 3512; Costar); monolayers attained confluency after 2 d at 37°C in a 7% CO₂ humidified atmosphere. 100 μ l of the appropriate dilution was added to each monolayer and incubated at room temperature for 30 min. Each well then received 1.5 ml of agar overlay medium containing MEM, 1.5% BSA, 2% amino acids (320–1130; Gibco Laboratories), 1% vitamins (320–1120; Gibco Laboratories), 2 mM glutamine (320–5030; Gibco Laboratories), antibiotics, 1% agar (Difco Laboratories, Inc., Detroit, MI), and 0.5 μ g/ml trypsin-TPCK (Worthington Biochemical Corp., Freehold, NJ). After 2 d incubation in 7% CO₂ at 37°C , 0.5 ml of a second agar overlay containing 0.01% neutral red, but no trypsin, was added to each well. The plates were incubated for 18 h and plaques were then counted. Each titration point of an individual lung extract was assayed in duplicate. Results are expressed as the mean plaque-forming units (PFU)/ml \pm SEM in lung extracts from three mice.

In the case of dual infection (simultaneous infection of mice with the A/JAP/57 and A/MEL/35 viruses), in order to quantitate the level of each virus in the lungs, the lung extracts were treated with anti-A/MEL/35 antiserum or anti-A/JAP/57 HA antiserum as described above before plaquing. Controls consisted of untreated extracts.

Assay for Cell-mediated Cytotoxicity. The ⁵¹Cr-release cytotoxicity assay was carried out as described (15). Briefly, 1×10^4 ⁵¹Cr-labeled uninfected or influenza virus-infected P815 target cells were added to individual wells of 96-well, flat-bottom microtiter tissue culture plates in a 0.1 ml vol of MEM + 10% FBS. Effector cells were cloned CTL and were added in a 0.1 ml vol of MEM + 10% FBS to each well. The plates were incubated at 37°C in 7% CO₂ for 6 h. From each well, 0.1 ml of supernatant was removed and counted in a Beckman 4000 Gamma Counter (Beckman Instruments, Inc., Fullerton, CA). The percent specific ⁵¹Cr release was calculated from the formula: [(test counts –

spontaneous release)/(total counts - spontaneous release)] \times 100. Spontaneous release from target cells incubated with medium alone usually ranged from 8 to 15% of total counts. Total release was determined by target cell solubilization in 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO). Release values represent the mean percent specific ^{51}Cr release from four replicate wells. SEM were always $<5\%$ of the mean value and are omitted.

Assays of Cellular Proliferation. Two methods were used to assess the proliferative response of the cloned CTL lines: direct estimates of cell concentration in stationary phase (day 5 of culture) and [^3H]thymidine ([^3H]TdR) incorporation at the peak of the proliferative response (day 3 of culture). Cell lines were subcultured as described above. 6-7 d after antigenic stimulation, cloned cells were resuspended in fresh medium containing 10% (vol/vol) TCGF. After 7 d in culture, 1×10^4 viable cells were plated into individual wells of 96-well flat-bottom tissue culture plates (Linbro, Flow Laboratories, Inc., Hamden, CT), along with 10^6 irradiated, uninfected or virus-infected BALB/c spleen cells in 0.2 ml of RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 5×10^{-5} M 2-ME, antibiotics, 10 mM Hepes, and 10% (vol/vol) TCGF. At 3, 4, and 5 d of culture at 37°C , the proliferative response of quadruplicate cultures was assessed by adding to each well 0.1 ml of PBS containing 1 μCi [^3H]TdR. After 4-h incubation at 37°C , individual wells were harvested onto glass fiber filters by using an automated harvester. Radioactivity was detected by standard scintillation methods. Results are expressed as the mean cpm \pm SEM of quadruplicate cultures. Viable cell concentrations in companion microwells were determined by trypan blue exclusion.

Cell Surface Phenotyping. The expression of Thy-1, Lyt-1, and Lyt-2 on the surface of these clones was detected using monoclonal reagents as described in detail elsewhere (14).

Results

Influenza Virus Specificity of CTL Clones In Vitro. The two cloned CTL populations used in this study, A4 and A7, were selected by limiting dilutions from an in vitro tertiary culture of spleen cells derived from an A/JAP/57 influenza virus-immunized BALB/c mouse. The clones were isolated, expanded, and maintained in the presence of A/JAP/57 virus-infected irradiated BALB/c spleen cells and 10% crude interleukin 2 (IL-2)-conditioned medium of rat spleen cell origin. Cell surface phenotyping (14) of these clones revealed that both clones are Thy-1.2 $^+$, Lyt-1-2 $^+$ (data not shown). These two clones of H-2 d origin were restricted by H-2 d gene products in the recognition of A/JAP/57 virus both at the level of target cell lysis and antigen-dependent proliferation. Patterns of H-2-end restriction of the clones were determined by the ability of the CTL clones to lyse a panel of A/JAP/57 virus-infected L929 (H-2 k) cells transfected with either the H-2K d , H-2L d , or H-2D d gene (unpublished observations). In this manner, A4 and A7 were determined to be restricted by H-2K d and H-2L d , respectively. At the time of this study, both cloned lines had been carried in continuous culture for over one year without any changes in morphology, growth characteristics, surface phenotype, or viral antigen specificity.

The viral antigen specificities of the A4 and A7 clones at the level of target cell recognition is shown in Table I. A4 recognizes an antigenic determinant shared by type A influenza viruses of the H2N2 subtype, e.g., A/JAP/57 and A/AA/67, and exhibits no cytotoxic activity against cells infected with either A/MEL/35 (H1N1 subtype) or A/HK/68 (H3N2 subtype). This is the typical pattern of subtype-specific CTL recognition reported previously (14). In contrast, A7 is directed to a viral antigen determinant common to type A influenza virus strains of different subtypes, e.g., A/JAP/57 (H2N2), A/MEL/35 (H1N1), and

TABLE I
Influenza Virus Specificity of CTL Clones

CTL line	Effector/ target ratio [‡]	Percent specific ⁵¹ Cr release from target cells*					B/Lee
		Unin- fected	A/JAP/57 (H2N2)	A/AA/67 (H2N2)	A/MEL/35 (H1N1)	A/HK/68 (H3N2)	
A4	0.5:1	0	48	35	0	1	0
	1:1	0	66	52	2	2	1
	2:1	0	84	67	2	2	1
A7	0.5:1	0	52	58	57	43	0
	1:1	0	76	76	77	54	0
	2:1	0	85	88	89	62	1

CTL lines were examined for cytotoxic activity on uninfected and infected ⁵¹Cr-labeled target cells 5 d after routine subculturing in the presence of 10% TCGF-supplemented medium and A/JAP/57 virus-infected irradiated BALB/c splenocytes.

* Values are the means from four replicate wells with spontaneous release subtracted. Spontaneous release from all target groups was <13%. SEM are <5% of mean values and are omitted.

[‡] 1 × 10⁴ P815 (H-2^d) target cells were added per well.

TABLE II
Viral Antigen and Growth Factor Dependence of CTL Clone Proliferation

BALB/c stimulator spleen cells in- fected with:*	Final cell concentration (× 10 ⁶ /ml) [‡]				Peak proliferative response [§]			
	A4 [¶]		A7		A4		A7	
	+	-	+	-	+	-	+	-
—	0.7	0.1	2.5	<0.1	221 ± 11	60 ± 10	1,200 ± 119	41 ± 2
A/JAP/57	7.4	0.3	16.8	0.1	6,286 ± 75	59 ± 3	34,961 ± 1,626	362 ± 21
A/AA/67	8.1	0.1	18.3	2.0	15,225 ± 220	95 ± 13	53,344 ± 1,334	2,831 ± 331
A/MEL/35	1.0	<0.1	17.5	0.6	1,106 ± 151	52 ± 4	22,697 ± 1,215	1,534 ± 240
A/HK/68	1.0	0.1	16.8	0.3	363 ± 49	56 ± 4	25,213 ± 1,176	391 ± 43
B/Lee	1.3	0.5	4.8	0.1	784 ± 35	41 ± 2	4,802 ± 310	44 ± 8

CTL lines A4 and A7 were subcultured in medium containing 10% crude TCGF and irradiated BALB/c spleen cells infected with A/JAP/57 virus. After 6 d of culture, cells of each line were resuspended in fresh medium containing 10% crude TCGF and cultured for an additional 7 d. After this time, replicate cultures of 10⁶ viable cells were analyzed for proliferation by [³H]TdR incorporation and direct cell counting after culturing under the indicated conditions.

* Normal BALB/c spleen cells after 2,000 rad of irradiation were either infected with the indicated virus strain or left uninfected. 1 × 10⁶ cells were co-cultured with 1 × 10⁴ cloned CTL cells as described (Materials and Methods).

[‡] Values are the viable cell concentrations at day 5 of culture. The starting cell concentration (day 0) was 0.5 × 10⁶/ml.

[§] Values are the mean cpm ± SEM of [³H]TdR incorporation from quadruplicate cultures at the peak of the proliferative response (day 3).

[¶] Cells of the indicated clone were cultured with the indicated splenocyte stimulator either in the presence (+) or absence (-) of 10% crude TCGF.

A/HK/68 (H3N2), and has the characteristic pattern of viral antigen recognition of cross-reactive CTL clones (14). As reported previously (19), the specificity of IL-2-dependent, antigen-specific proliferation of these clones in vitro directly parallels their cytolytic effector specificity (Table II). Thus, A4 proliferates significantly only in response to stimulation by A/JAP/57 virus- or A/AA/67 virus-infected, irradiated BALB/c spleen cells. A7, on the other hand, proliferates in the presence of irradiated BALB/c spleen cells infected with viruses of either of the three subtypes represented here. Importantly, both clones were dependent on both antigen and exogenous IL-2 for proliferation; i.e., neither

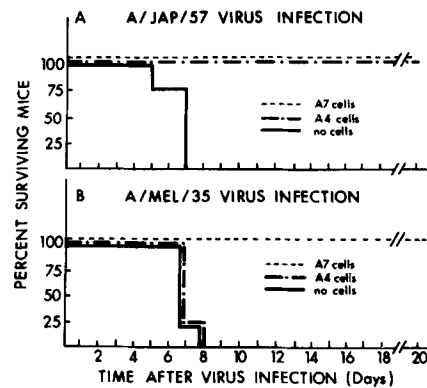


FIGURE 1. Viral antigenic specificity of in vivo antiviral effector activity of CTL clones A4 and A7. BALB/c mice were inoculated intranasally with 10 LD_{50} of either A/JAP/57 virus (A) or A/MEL/35 virus (B). Within 1 h of infection, groups of four mice each received either 1×10^7 cells of clone A7 (---), 1×10^7 cells of clone A4 (—), or were left untreated (-·-). Cumulative mortality was tabulated for 20 d.

A4 nor A7 proliferates to a specific antigenic stimulation in the absence of exogenous IL-2.

In Vivo Effector Function of Cloned CTL Lines. To assess the in vivo effector function of CTL clones, the following protocol was used. Panels of syngeneic or allogeneic mice were inoculated with 10 LD_{50} of infectious virus by the intranasal route. 1 h after lethal infection, 10^7 cloned cells were adoptively transferred into test recipients intravenously via the tail vein. Control infected recipients received medium with no cells. These test and control recipients were monitored for overall survival and pulmonary virus titer as described below. This overwhelming lethal inoculating virus dose was used to minimize the effect of the endogenous host response on the outcome of infection. As described below (Fig. 1), both the A/JAP/57 and A/MEL/35 virus infections were uniformly lethal by 7–10 d postinoculation.

Since previous reports had demonstrated that after adoptive transfer, heterogeneous immune cell populations possessing cytotoxic activity protected mice from lethal primary influenza virus pneumonia, our initial experiments sought to verify that cloned CTL could mediate this antiviral effect in vivo. As shown in Fig. 1A, both the subtype-specific A4 clone and the cross-reactive A7 clone completely protected mice from death by A/JAP/57 (H2N2) influenza virus, the virus subtype used to select and maintain the clones in vitro. In contrast, while the cross-reactive A7 clone promoted recovery of recipients from lethal infection with A/MEL/35 (H1N1) virus, the subtype-specific clone A4 had no effect on the lethal outcome of A/MEL/35 infection after adoptive transfer (Fig. 1B). Neither clone altered the outcome of lethal infection with the unrelated B/Lee virus and the in vivo protective effect of the clones is H-2 restricted (Lukacher, Braciale, and Braciale, manuscript in preparation). Thus, the viral antigenic specificity exhibited by these CTL clones in vitro in cytotoxicity (Table I) and proliferation assays (Table II) precisely parallels the specificity of their in vivo capacity to confer protection from lethal primary influenza pneumonia (Fig. 1).

The association of recovery from primary influenza pneumonia with falling

TABLE III
Specificity of Pulmonary Virus Reduction by CTL Clone A4

Cloned CTL cells transferred*	Recipients	
	Virus instilled intranasally [‡]	Virus titer in lungs [§]
None	A/JAP/57 (H2N2)	$(1.61 \pm 0.30) \times 10^6$
A4	A/JAP/57	<100
None	A/MEL/35 (H1N1)	$(2.07 \pm 0.52) \times 10^6$
A4	A/MEL/35	$(2.00 \pm 0.45) \times 10^6$

* 1×10^7 cells of cloned CTL line A4 were injected intravenously into each BALB/c recipient at the same time as virus infection. Control infected mice received 0.5 ml medium i.v.

[‡] Recipient mice were inoculated intranasally with 10 LD₅₀ of the indicated influenza type A virus strain. Virus subtype appears in parentheses.

[§] Expressed as mean PFU/ml \pm SEM of lung extracts from three individual mice at day 5 after infection as assayed by plaque formation on MDCK cell monolayers (Materials and Methods).

pulmonary virus titers has been well documented (3, 5, 20). In light of the failure of clone A4 to protect across subtypes, it was of interest to correlate protection by A4 with reduction in pulmonary virus titers in A/JAP/57- and A/MEL/35-infected recipients. Infectious virus particles in mouse lung extracts were titered by plaque formation on monolayers of MDCK cells. In preliminary studies, a kinetic analysis of lung virus levels in mice infected with 10 LD₅₀ of A/JAP/57 virus which received 10^7 cloned CTL cells i.v. demonstrated that pulmonary virus titers progressively decreased from 24 to 96 h after infection, with a 100–1,000-fold reduction in infectious particles attained by 96 h after infection. No virus was detectable in lungs of recovered mice at 3 wk postinfection, the end of the observation period for the protection studies described above (data not shown). Based on these considerations, for the present studies pulmonary virus titers were measured 5 d postinfection. Table III shows the pulmonary virus titer at 5 d postinfection with A/JAP/57 or A/MEL/35 of control recipients and recipients of 10^7 adoptively transferred A4 cells administered at day 0. Transfer of clone A4 is associated with a >1,000-fold reduction in virus titer from the lungs of A/JAP/57-infected recipients, but clone A4 has no effect on the pulmonary viral titer in A/MEL/35-infected recipients. As described below (Table IV), clone A7 efficiently reduces the pulmonary virus titer of both A/JAP/57- and A/MEL/35-infected recipients. Hence, pulmonary virus titer correlates with the recovery of cloned cell recipients, and both survival data and pulmonary viral titers demonstrate the subtype-specific behavior of clone A4 in vivo.

In Vivo Effects of Cloned CTL Lines in Mice Simultaneously Infected with A/JAP/57 and A/MEL/35 Viruses. The subtype-specific effector activity of clone A4 in vivo afforded the opportunity to examine the specificity of the antiviral effector mechanism used to eliminate virus and promote recovery in vivo. Since specific antigenic recognition appears to be necessary to trigger lymphokine release by CTL (11, 12), it is possible that soluble mediators released by specific induction with antigen could act nonspecifically to promote recovery. To examine this issue, panels of mice were simultaneously infected with 10 LD₅₀ of both A/JAP/

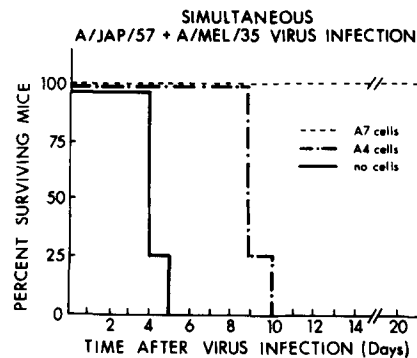


FIGURE 2. Ability of cloned CTL lines A4 and A7 to promote recovery of mice infected simultaneously with virus strains of two different type A influenza virus subtypes. Protocol is as described in Fig. 1, except that recipients were inoculated simultaneously with 10 LD₅₀ A/JAP/57 virus and 10 LD₅₀ A/MEL/35 virus.

57 (H2N2) and A/MEL/35 (H1N1) virus and then inoculated with either clone A4 (H2N2 subtype specific) or A7 (type A cross-reactive) according to the above protocol. The survival curves for these various groups are shown in Fig. 2. Control mice that were dually infected but received no cells had a slightly accelerated mortality (time to death, 4–6 d) compared with either lethally infected A/JAP/57 recipients (time to death, 6–8 d) or A/MEL/35 recipients (time to death, 8–10 d). Recipients of the cross-reactive A7 clone, on the other hand, were completely protected from the lethal dual infection. Most importantly, subtype-specific clone A4 failed to promote recovery of these dual-infected recipients whose mortality curve was similar to the curve of A/MEL/35 infection (Fig. 1B).

The exquisite specificity of the antiviral effector activity exhibited by A4 *in vivo* was further substantiated by measurements of pulmonary virus titers at day 5 after simultaneous A/JAP/57 and A/MEL/35 infection. As shown in Table IV, clone A4 dramatically decreased the level of infectious A/JAP/57 virus by >1,000-fold relative to controls but failed to reduce the titer of infectious A/MEL/35 in these dual-infected lungs. Thus, the clone is able to efficiently eliminate one virus in the face of high levels of another related but antigenically distinct virus. On the other hand, A7 not only reduces A/JAP/57 pulmonary titers to the same extent as A4, but also decreases A/MEL/35 virus levels ~2 log₁₀ units below that of control mice. Had pulmonary virus titrations for the group that received A7 been determined at time points beyond 5 d postinfection, complete elimination of A/MEL/35 would have been achieved. The fact that this group recovered from this dual infection supports this claim. Furthermore, the gross appearance of lungs of these mice at 3 wk postinfection was indistinguishable from those of normal mice, further suggesting that infectious virus had been eliminated before the development of irreversible pulmonary damage.

Discussion

In this report we have demonstrated that cloned populations of influenza virus-specific CTL maintained in continuous long-term culture (>1 yr) can

TABLE IV
Pulmonary Virus Titer Reduction Mediated by CTL Clones in Mice Simultaneously Infected With Influenza Type A Virus Strains of Different Subtypes

Cloned CTL cells transferred	Viral specificity of cloned CTL line	Pulmonary Virus Titer of Dually Infected Recipients*		
		Total Virus (A/JAP + A/MEL) [‡]	A/JAP [§]	A/MEL [¶]
None	—	$(2.0 \pm 0.6) \times 10^6$	$(4.8 \pm 1.4) \times 10^5$	$(1.2 \pm 0.5) \times 10^6$
A4	Subtype specific	$(1.8 \pm 0.5) \times 10^6$	<100	$(2.0 \pm 0.1) \times 10^6$
A7	Cross-reactive	$(2.0 \pm 1.0) \times 10^4$	<100	$(2.5 \pm 1.4) \times 10^4$

Recipient mice were simultaneously inoculated with 10 LD₅₀ A/JAP/57 virus and 10 LD₅₀ A/MEL/35 virus intranasally. Immediately after infection, 1×10^7 cells of either clone A4 or clone A7 were injected intravenously into panels of infected recipients. Controls consisted of dually infected mice that had received no cells.

* Lung extracts prepared from recipients 5 d after infection were treated for 1 h at 4°C with rabbit anti-A/MEL/35 antiserum, goat anti-A/JAP/57 HA antiserum, or no antiserum, before virus titration of MDCK cell monolayers.

[‡] Indicates total infectious virus titer expressed as mean PFU/ml \pm SEM of lung extracts from three dually infected mice per value.

[§] Residual infectious virus titer after treatment with neutralizing anti-A/MEL/35 antiserum.

[¶] Residual infectious virus titer after treatment with neutralizing anti-A/JAP/57 HA antiserum.

mediate recovery from lethal influenza virus infection. The viral specificity of in vivo protection by the clones directly corresponds to their in vitro specificity as defined in cytotoxicity and proliferation assays. Thus, specific antigenic recognition appears to be required for the expression of in vivo antiviral activity. Furthermore, the protective effect of the cloned CTL is associated with the specific elimination of infectious virus from the lungs of recovering animals. This result suggests a direct effect of the CTL on the viral infection process.

A major finding in this study is the observation that the H2N2 subtype-specific clone A4 is unable to alter the lethal outcome of simultaneous infection with the A/JAP/57 (H2N2) and A/MEL/35 (H1N1) viruses. In these dually infected recipients, A4 reduced the pulmonary titers of A/JAP/57 virus by at least three orders of magnitude but the clone failed to decrease the titer of the A/MEL/35 virus. The clone therefore was able to selectively inhibit the replication of the specifically recognized influenza strain without affecting the titer of the irrelevant influenza virus strain. Also, the mortality curve for this dual infection group was similar to that of A/MEL/35-infected recipients of clone A4. This strongly suggests that the A4 recipient mice had eliminated the A/JAP/57 virus and died of A/MEL/35 pneumonia. In contrast, the cross-reactive A7 clone reduced the pulmonary titers of both viruses in the dual-infected recipients and promoted complete recovery.

These results have two related implications. First, they imply that in this experimental system the CTL clones are probably not expressing their antiviral effect in vivo primarily by orchestrating nonspecific host defense mechanisms in the recipients. More importantly, these results favor the concept that CTL mediate their antiviral protective effect in vivo via direct cytolysis of virus-infected cells. Although we cannot at present rule out the involvement of short-lived soluble mediators released by the clones in high local concentrations, it

seems highly unlikely that the antiviral effect of the clones *in vivo* is due to antigen-nonspecific lymphokines, e.g., interferon- γ , produced by the CTL after specific antigenic stimulation. In this connection, it should be noted that both clones can secrete interferon after stimulation with mitogenic lectins or virus-infected target cells (unpublished observations).

It is of considerable interest that these protective CTL clones require both specific antigenic stimulation and an exogenous source of IL-2 for their *in vitro* propagation. This observation is at variance with the findings of Engers et al. (21). These investigators showed that alloreactive CTL clones dependent on antigen and exogenous growth factors for *in vitro* growth failed to express *in vivo* effector activity, whereas autonomously proliferative CTL clones (22, 23) were effective *in vivo*. In our hands, both autonomous and exogenous IL-2-dependent anti-influenza CTL clones showed similar levels of antiviral activity *in vivo* (unpublished observations). Furthermore, in preliminary studies, adoptive transfer of limited numbers of these IL-2-dependent CTL clones after lethal irradiation to inhibit proliferation did result in prolonged survival of lethally infected recipients, but complete recovery of all recipients did not occur. It is therefore possible that these antigen- and exogenous growth factor-dependent clones need not proliferate *in vivo* to express an antiviral effect.

Another consideration thought to explain the success or failure of cloned CTL to exhibit effector activity *in vivo* is altered cell trafficking of these *in vitro* propagated clones, most notably nonspecific trapping of cells in the pulmonary vasculature (24). At present we do not believe that the expression of *in vivo* effector activity by the clones reported here is due to nonspecific trapping of the clones at the site of antigenic sequestration, the infected lung. Cell trafficking studies using autoradiographic detection of biosynthetically labeled clone cells indicate that the cells leave the pulmonary vasculature and migrate to the pulmonary parenchyma and alveolar spaces of both infected and uninfected mice (unpublished observations).

A number of host defense mechanisms, both specific and nonspecific, have been implicated as antiviral effectors in protection and recovery from infection (25). In experimental models of viral infection, the importance of a particular defense mechanism in recovery appears to depend on both the type of virus and the severity of infection. For example, natural killer cell activity has been demonstrated to have a significant antiviral effect on infection with some viruses but not others (26, 27). The data presented here strongly suggest that antiviral CTL represent an extremely potent effector mechanism at least for influenza infection. Even under the conditions of overwhelming infection used for these studies, as few as $1-2 \times 10^6$ cloned CTL could prolong survival and promote recovery of a significant percentage of lethally infected recipients (A. E. Lukacher, unpublished observations). It is anticipated that, with infection at minimal lethal virus doses, perhaps as few as 10^5 cells or less of a particular clone could be protective *in vivo*. Studies detailing the antiviral effect of adoptively transferred CTL clones will be needed in a number of different viral systems to better define the role of this T cell subset in recovery from infection.

In conclusion, we have demonstrated that cloned populations of influenza-specific CTL propagated in the presence of specific antigen and an exogenous

IL-2 source can efficiently eliminate infectious virus from the lungs of lethally infected mice and promote recovery. Both the induction and expression of antiviral activity by the CTL clones in vivo is highly specific from the standpoint of viral antigen recognition. It therefore seems likely that direct cytolysis of virally infected cells is the primary mechanism by which CTL express their antiviral effector activity in vivo. The availability of cloned CTL with in vivo functional activity should now enable study of the molecular basis of the in vivo effects of CTL.

Summary

Cloned lines of murine cytotoxic T lymphocytes (CTL) directed to type A influenza virus confer complete protection upon adoptive transfer to syngeneic mice lethally infected by influenza virus. The exquisite specificity exhibited by a subtype-specific cloned CTL in culture is reflected in its capacity to eliminate pulmonary virus and mediate recovery only in those mice infected by the virus subtype recognized by this cloned line in vitro. A cross-reactive CTL cloned line protects mice infected by either of two influenza virus subtypes. In mice dually infected with two virus subtypes, the subtype-specific CTL clone only reduces lung virus levels of the recognized virus subtype and cannot prevent these mice from dying. In contrast, adoptive transfer of the cross-reactive CTL clone into mice simultaneously infected with two virus subtypes results in reduction of pulmonary titers of both subtypes and promotes complete recovery. These results directly implicate CTL as an important antiviral defense mechanism in experimental influenza infection. In addition, these results indicate that both the induction and expression of antiviral effector activity by CTL in vivo is highly specific and therefore favor the concept that CTL express their antiviral effect in vivo by direct cytolysis of infected cells.

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References

1. Zinkernagel, R. M., and R. M. Welsh. 1976. H-2 compatibility requirement for virus-specific T cell-mediated effector function in vivo. I. Specificity of T cells conferring antiviral protection against lymphocytic choriomeningitis virus is associated with H-2K and H-2D. *J. Immunol.* 117:1495.
2. Blanden, R. V. 1977. Cell-mediated immune response to acute viral infection. *In* Progress in Immunology III. T. E. Mandel, C. Cheers, C. S. Hosking, I. F. C. McKenzie, and G. J. V. Nossal, editors. North Holland Publishing Company, Amsterdam. 463-471.
3. Yap, K. L., and G. L. Ada. 1978. Cytotoxic T cells in the lungs of mice infected with an influenza A virus. *Scand. J. Immunol.* 7:73.
4. Yap, K. L., T. J. Braciale, and G. L. Ada. 1979. Role of T cell function in recovery from murine influenza infection. *Cell. Immunol.* 43:341.

5. Yap, K. L., and G. L. Ada. 1978. The recovery of mice from influenza virus infection: adoptive transfer of immunity with immune T lymphocytes. *Scand. J. Immunol.* 7:389.
6. Wells, M. A., F. A. Ennis, and P. Albrecht. 1981. Recovery from a viral respiratory infection. II. Passive transfer of immune spleen cells to mice with influenza pneumonia. *J. Immunol.* 126:1042.
7. Yap, K. L., G. L. Ada, and I. F. C. McKenzie. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature (Lond.)*. 273:238.
8. Lin, Y. L., and B. A. Askonas. 1981. Biological properties of an influenza A virus-specific killer T cell clone: inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. *J. Exp. Med.* 154:225.
9. Taylor, P. A., and B. A. Askonas. 1983. Diversity in the biological properties of anti-influenza cytotoxic T cell clones. *Eur. J. Immunol.* 13:707.
10. Prystowsky, M. B., J. M. Ely, D. I. Beller, L. Eisenberg, J. Goldman, M. Goldman, E. Goldwasser, J. Ihle, J. Quintans, H. Remold, S. N. Vogel, and F. W. Fitch. 1982. Alloreactive cloned T cell lines. IV. Multiple lymphokine activities secreted by helper and cytolytic cloned T lymphocytes. *J. Immunol.* 129:2337.
11. Morris, A. G., Y. L. Lin, and B. A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T cell line meets its correct influenza-infected target cell. *Nature (Lond.)*. 295:150.
12. Klein, J. R., D. H. Raulet, M. S. Pasternack, and M. J. Bevan. 1982. Cytotoxic T lymphocytes produce immune interferon in response to antigen or mitogen. *J. Exp. Med.* 155:1198.
13. Zinkernagel, R. M., and A. Althage. 1977. Antiviral protection by virus-immune cytotoxic T lymphocytes: infected target cells are lysed before infectious virus progeny is assembled. *J. Exp. Med.* 145:644.
14. Braciale, T. J., M. E. Andrew, and V. L. Braciale. 1981. Heterogeneity and specificity of cloned lines of influenza virus-specific cytotoxic T lymphocytes. *J. Exp. Med.* 153:910.
15. Braciale, T. J. 1977. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus strain-specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell. Immunol.* 33:423.
16. Braciale, T. J. 1977. Immunologic recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells. *J. Exp. Med.* 146:673.
17. Irwin, J. O., and E. A. Cheeseman. 1939. On an appropriate method of determining the median effective dose and its error in the case of a quantal response. *J. Hyg.* 39:574.
18. Davidson, W. F., and C. R. Parish. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J. Immunol. Methods.* 7:291.
19. Andrew, M. E., and T. J. Braciale. 1981. Antigen-dependent proliferation of cloned continuous lines of H-2-restricted influenza virus-specific cytotoxic T lymphocytes. *J. Immunol.* 127:1201.
20. Wells, M. A., P. Albrecht, and F. A. Ennis. 1981. Recovery from a viral respiratory infection. I. Influenza pneumonia in normal and T-deficient mice. *J. Immunol.* 126:1036.
21. Engers, H. D., A. L. Glasebrook, and G. D. Sorenson. 1982. Allogeneic tumor rejection induced by the intravenous injection of Lyt-2⁺ cytolytic T lymphocyte clones. *J. Exp. Med.* 156:1280.
22. Widmer, M. B., and F. H. Bach. 1981. Antigen-driven helper cell-independent cloned cytolytic T lymphocytes. *Nature (Lond.)*. 294:750.

23. Glasebrook, A. L., A. Kelso, and H. R. MacDonald. 1983. Cytolytic T lymphocyte clones that proliferate autonomously to specific alloantigenic stimulation. II. Relationship of the Lyt-2 molecular complex to cytolytic activity, proliferation, and lymphokine secretion. *J. Immunol.* 130:1545.
24. Lotze, M. T., B. R. Line, D. J. Mathisen, and S. A. Rosenberg. 1980. The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): implications for the adoptive immunotherapy of tumors. *J. Immunol.* 125:1487.
25. Mims, C. A. 1982. *The Pathogenesis of Infectious Disease*. Academic Press, Inc., New York. Second ed. 199–222.
26. Shellam, G. R., J. E. Allan, J. M. Papadimitriou, and G. J. Bancroft. 1981. Increased susceptibility to cytomegalovirus infection in beige mutant mice. *Proc. Natl. Acad. Sci. USA.* 78:5104.
27. Bukowski, J. F., B. A. Woda, S. Habu, K. Okumura, and R. M. Welsh. 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. *J. Immunol.* 131:1531.